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ACETALDEHYDE AND ALCOHOLIC BEVERAGES IN UPPER DIGESTIVE TRACT CANCER – REDUCTION OF EXPOSURE WITH CYSTEINE

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Potential mechanism for calvados related oesophageal cancer; Klas Linderborg, Jean Pierre Joly, Jukka-Pekka Visapää and Mikko Salaspuro; Food. Chem. Toxicol. 2008 Feb;46(2):476-479

II. A single sip of a strong alcoholic beverage causes exposure to carcinogenic concentrations of acetaldehyde in the oral cavity; Linderborg K, Salaspuro M, Väkeväinen S; Food. Chem. Toxicol. 2011 Sep;49(9):2103-6.

III. Reducing carcinogenic acetaldehyde exposure in the achlorhydric stomach with cysteine; Linderborg K, Marvola T, Marvola M, Salaspuro M, Färkkilä M, Väkeväinen S; Alcohol. Clin. Exp. Res. 2011 Mar;35(3):516-522

ABBREVIATIONS

AC	adenocarcinoma
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AUC	area under the curve
BAC	blood alcohol content
BMI	body mass index
bw	body weight
CI	confidence interval
ESCC	oesophageal squamous cell carcinoma
GI	gastrointestinal
HPLC	high performance liquid chromatography
HNC	head and neck cancer
HPV	human papilloma virus
IARC	International Agency for Research on Cancer
K_m	Michaelis constant
MEOS	microsomal ethanol oxidizing system
MTCA	2-methyl-4-thiazolidine-carboxylic acid
NAD	nicotineamide adenine dinucleotide
NADP	nicotineamide adenine dinucleotide phosphate
NMTCA	N-nitroso-2-methyl-4-thiazolidine-carboxylic acid
OR	odds ratio
PAR	population attributable ratio
SCC	squamous cell carcinoma
UADT	upper aerodigestive tract
WHO	World Health Organization

ABSTRACT

Background

There is clear evidence that alcohol consumption is a risk factor for several cancers in humans. Ethanol is not carcinogenic as a molecule, but there is conclusive evidence that acetaldehyde, formed by microbial metabolism of ethanol in the upper digestive tract, acts there as a local carcinogen. Acetaldehyde is found in varying concentrations in alcoholic beverages and also in foods. This acetaldehyde could contribute to overall acetaldehyde exposure in the upper digestive tract. Consumption of calvados has been linked to an increased risk for oesophageal cancer in France. Achlorhydric gastritis is a premalignant condition, in which gastric bacterial overgrowth can lead to increased acetaldehyde concentrations in the stomach after ethanol ingestion. L-Cysteine binds to acetaldehyde and can be used to lower acetaldehyde concentrations possibly reducing acetaldehyde exposure, and the carcinogenic effects thereof.

Aims

The first and second aim of this thesis was to examine acetaldehyde concentrations in calvados and other alcoholic beverages, and to study the exposure to acetaldehyde after a sip of these beverages. The third aim was to develop and test a slow-release L-cysteine formulation for eliminating carcinogenic acetaldehyde in achlorhydric stomach during ethanol exposure.

Methods

Firstly, farm-made calvados was collected in Normandy, France. Ethanol and acetaldehyde concentrations were measured and compared to samples of commercially available alcoholic beverages. Secondly, salivary acetaldehyde concentrations were measured after small sips of alcoholic beverages. Calvados with high acetaldehyde concentration was compared to ethanol of similar concentration without acetaldehyde. Thirdly slow-release L-cysteine capsules were formulated and given to volunteers with achlorhydric gastritis prior to infusion of dilute ethanol through nasogastric intubation. Samples of gastric juice were subsequently aspirated and analysed for acetaldehyde and L-cysteine concentration.

Results and conclusions

We found 42% higher mean acetaldehyde concentrations in farm-made and industrially manufactured calvados when compared to other alcoholic beverages. Markedly elevated concentrations of acetaldehyde were found to be produced from ethanol in the oral cavity instantly after a small sip of alcoholic beverage, and that the exposure continued for at least 10 minutes. Acetaldehyde present in the beverage had a small, short-term increasing effect on total acetaldehyde exposure. Furthermore, we found that L-cysteine can be used to decrease acetaldehyde concentration to less than half in gastric juice after ethanol ingestion in test subjects with achlorhydric gastritis.

Acetaldehyde produced microbially from ingested ethanol is probably the main source for carcinogenicity of ethanol in upper digestive tract, although acetaldehyde in beverages contributes slightly to overall acetaldehyde exposure. This could explain the increased risk for oesophageal cancer associated with consumption of hot calvados. Slow-release L-cysteine capsules can be used to reduce acetaldehyde exposure in achlorhydric stomach during ethanol consumption.

INTRODUCTION

The WHO estimates that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide. The worldwide risk of getting vs. dying of cancer before the age of 75 is 18.5% vs. 10.4%, respectively (IARC 2018). Alcohol consumption increases the risk of oral, pharyngeal, laryngeal, oesophageal, gastric, liver, colorectal and female breast cancer (Boffetta, Hashibe 2006, Bagnardi et al. 2015). It has been estimated that globally 5.5% of all cancers (7.2% in men, 3.5% in women) are attributable to alcohol drinking (Praud et al. 2016).

In western Europe the figures are somewhat higher. Data from the prospective cohort study EPIC (The European Prospective Investigation into Cancer and Nutrition) showed that among men and women 10% and 3% of the incidence of all cancers were attributable to former and current alcohol consumption. The figures were even higher for selected cancers, 44% vs. 25% for upper aerodigestive tract cancer, 33% vs. 18% for liver cancer and 17% vs. 4% for colorectal cancer, for men and women respectively, and 5% for female breast cancer. The risks for all cancers related to alcohol consumption increased in a dose-dependent manner, with no lower limit below which the risk of cancer is decreased (Schutze et al. 2011). Accordingly, in a meta-analysis by Bagnardi et. al. even light drinking up to 1 drink/day compared to non-drinking is associated with a relative risk ratio of 1.17 for oropharyngeal, 1.30 for oesophageal squamous cell carcinoma and 1.05 for female breast cancer (Bagnardi et al. 2013).

In conclusion, alcohol drinking is associated with an increased risk for cancer, with no safe lower limit of consumption. There have been reports of increased cancer risk from certain types of alcoholic beverages, one of which gave the idea for the first original publication in this thesis (Launoy et al. 1997). However, analysis of epidemiological data available shows little indication that the carcinogenic effects of alcohol drinking depends on the type of beverage and it is concluded that ethanol itself is the determinant (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012).

As ethanol itself is not mutagenic the mechanism of carcinogenesis associated with alcohol drinking has to date not been fully elucidated. There is strong evidence that points to acetaldehyde, the first metabolite of ethanol, being the key ingredient in aerodigestive tract carcinogenesis (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012). Acetaldehyde is formed from ethanol locally in the upper digestive tract mainly by microbes representing normal oral flora (Homann et al. 1997).

Acetaldehyde is also present in varying amounts in different alcoholic beverages (Lachenmeier, Sohnius 2008). Furthermore, acetaldehyde is formed from ethanol in the achlorhydric stomach of test subjects with atrophic gastritis (Vakevainen et al. 2002), and also in healthy volunteers (Maejima et al. 2015).

The aims of this study were to investigate acetaldehyde content of different types of calvados, especially farm made calvados, and to determine the effect of sipping alcoholic beverages with different acetaldehyde content on salivary acetaldehyde concentration. Also, we wanted to determine whether L-cysteine can be used to deactivate carcinogenic acetaldehyde formed from ethanol in achlorhydric stomach.

REVIEW OF THE LITERATURE

Alcohol drinking and cancer - epidemiology

Oral and pharyngeal cancer

The IARC has summarized the data available on alcohol drinking and cancer up to 2009. Alcohol consumption increases the risk for oropharyngeal cancer in a dose-dependent manner. (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012). In a meta-analysis covering case-control and cohort studies up to October 2010 the risk ratio in non-smokers was 1.05-1.67 (95% CI) for drinking and 1.80-3.58 for heavy drinking (≥ 4 drinks/day). Concurrent smoking clearly increases the RR to 2.31-3.70 for overall drinking and 5.05-7.90 for heavy drinking. No significant difference in risk ratios for consumers of different types of beverages was found (Turati et al. 2013.). The type of beverage most frequently consumed within a population is usually the one associated with the highest risk (Boffetta, Hashibe 2006). The RR for light drinking (≤1 drink daily) was 1.17; 95% CI, 1.06-1.29 for oropharyngeal cancer (Bagnardi et al. 2013).

In a multicentre European case-control study, smoking and alcohol drinking were the most important risk factors for oropharyngeal cancer, with a population-attributable ratio (PAR) of 73.9% combined. The PAR was 0.3% for alcohol alone, 29.7% for smoking alone and 44.1% for alcohol drinking and smoking combined (Anantharaman et al. 2011). The EPIC study estimates a RR of 1.09; 95% CI, 1.06-1.12 (men) vs. 1.26; 95% CI, 1.07-1.49 (women) per every 10g daily increase in lifetime alcohol intake, when adjusted for smoking, and other life habits such as education, fruit and vegetable intake, body mass index and never- and former drinkers (Weikert et al. 2009).

Long-term frequent use of mouthwash – which typically contains alcohol in the order of 20% - is also associated with an increased risk for oral cancer (OR 1.28; 95% CI, 1.06-1.56 for use more than 35 years.) (Boffetta et al. 2015).

Laryngeal cancer

RR for laryngeal cancer was estimated at 2.65; 95% CI, 2.19-3.19 for heavy drinkers vs. non- or occasional drinkers in a recent comprehensive meta-analysis (Bagnardi et al. 2015). Again, the most frequently consumed alcoholic beverage tends to yield the highest risk in a population (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012). Light consumption of alcohol was not associated with a risk for laryngeal cancer (Bagnardi et al.

2013). In the EPIC study the dose dependence was estimated to a RR adjusted for smoking and other life habits of 1.08; 95% CI 1.05-1.12, for men and 1.32; 95% CI, 0.93-1.89 for women per every 10g daily increase in lifetime alcohol consumption (Weikert et al. 2009). Population attributable ratio of hypopharyngeal / laryngeal cancer was 0.02% for alcohol alone, 36.1% for smoking alone and 48.1% for alcohol drinking and smoking combined (Anantharaman et al. 2011).

Oesophageal cancer

Oesophageal cancer presents as squamous cell cancer (SCC) and adenocarcinoma (AC). The former of these is clearly associated with alcohol consumption. Bagnardi estimates the RR for oesophageal SCC at 1.26; 95% CI, 1.06-1.50 for light drinkers, 2.23; 95% CI, 1.87-2.65 for moderate drinkers and 4.95; 95% CI, 3.86-6.34 for heavy drinkers. The same data shows no risk from alcohol consumption for oesophageal AC or gastric cardia cancer. RR were 0.86; 95% CI,0.76-0.98, 0.97; 95% CI, 0.78-1.22 and 1.15; 95% CI, 0.95-1.39 respectively (Bagnardi et al. 2015). Dose dependence when adjusted for smoking and other life habits was estimated at RR for oesophageal SCC of 1.18; 95% CI, 1.10-1.27 and 1.35; 95% CI, 1.13–1.60 for men and women, respectively, per every 10g increase in daily alcohol intake (Weikert et al. 2009).

Oesophageal SCC is more common than AC in most populations with a ratio of 34:1 in African Americans to 1:1 in South Australian men. An increase in the incidence of AC has been observed, whereas the incidence of SCC has remained stable. The increase of AC has been attributed to an increase in obesity and gastrointestinal reflux (Vizcaino et al. 2002).

The risk factors for oesophageal SCC are tobacco use, alcohol consumption, mutations in enzymes that metabolise alcohol, achalasia, caustic injury, history of thoracic radiation, low socioeconomic status, poor oral hygiene, nutritional deficiencies, and non-epidermolytic palmoplantar keratoderma. Only a few of these are shared risk factors for oesophageal AC. The risk factors for oesophageal AC include symptomatic gastro-oesophageal reflux disease, Barrett´s oesophagus, obesity, tobacco use, history of thoracic radiation, diet low in vegetables and fruits, increased age, male sex, medications that relax the lower oesophageal sphincter, and rarely familial history (Pennathur et al. 2013). Pernicious anaemia and atrophic gastritis is also a risk factor for oesophageal SCC (Islami et al. 2011, Ye, Nyren 2003).

Drinking cessation leads to a slow decrease in oesophageal cancer risk, and it has been estimated that 16 years is required until all elevated risk has disappeared (Jarl, Gerdtham 2012).

Part of this thesis was inspired by discovery of an association between hot calvados and oesophageal cancer. When investigating regional differences in the incidence of oesophageal cancer in France it was found that consumption of hot calvados (as grog with hot water, or with hot coffee) was associated with an increased risk for oesophageal squamous cell cancer, when adjusted for life habits and socioeconomic status and consumption of other alcoholic beverages (OR = 2.33 for 41g alcohol / week; 95% CI, 1.12-4.87) (Launoy et al. 1997). Yet again in most studies the beverages most commonly consumed were associated with the highest risk for oesophageal cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012).

In the meta-analysis by Chen et. al. consumption of hot beverages and foods was also associated with oesophageal cancer, specifically SCC (OR 1.60; 95% CI, 1.29-2.00), and not AC (OR: 0.79; 95%CI, 0.53-1.16) There was however no significant association in a European population (OR 0.95; 95%CI, 0.68-1.34), whereas risks were significant in an Asian population (OR 2.06; 95% CI, 1.62-2.61) and a South American population (OR 1.52; 95% CI, 1.25-1.85) (Chen et al. 2015).

Gastric cancer

Helicobacter Pylori infection is the single most important risk factor for gastric cancer accounting for between 65-80% of non-cardia gastric cancers (Helicobacter and Cancer Collaborative Group 2001). The association between alcohol consumption and gastric cancer is not as clear as in other upper digestive tract cancers. The IARC concluded in 2012 that data on the association of alcohol drinking and gastric cancer is inconsistent, and pointed out that in no studies was it possible to adjust fully for lifetime infection with *H. Pylori* (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012).

In a study from South Korea drinking ≥ 7 times a week or drinking ≥ 55g alcohol per occasion was associated with an increased risk for gastric cancer in *H. Pylori* negative individuals (OR 3.48; 95% CI, 1.13-10.73 and 3.27; 95% CI, 1.01-10.56), whereas there was no increased risk in *H. Pylori* positive individuals (OR 1.17; 95% CI, 0.78-1.77 and 0.94; 95% CI, 0.61-1.46). It is noted that the No. of cancer cases in the *H. Pylori* negative group was only 31 vs. 235 in the *H. Pylori* positive group (Ma, S. H. et al. 2015).

There is also evidence of an increased risk for gastric cancer associated with allelic variants in the ADH1 gene cluster from the EPIC data, and the risk was accentuated by increased alcohol, and especially beer consumption (Duell et al. 2012). This suggests an involvement of alcohol metabolism in gastric cancer risk.

Light or moderate alcohol drinking does not seem to be a risk factor for gastric cancer. A meta-analysis of available data from 2012 with 44 case-control, and 15 cohort studies estimated the RR at 1.07; 95% CI, 1.01-1.13 for alcohol drinkers vs. non-drinkers, and at 1.20; 95% CI, 1.01-1.44 for heavy drinkers (Tramacere et al. 2012).

In a more recent meta-analysis by Ma et al. including 10 case-control studies an increased risk for gastric cancer was found when compared to non-drinkers for moderate drinkers (OR 1.30; 95%CI, 1.13-1.50) and heavy drinkers (OR 1.58; 95%CI, 1.21-2.05). Moderate drinking was defined as 15 grams a day for women, and 30 grams a day for men. Heavy drinking was defined as more than 30 grams alcohol a day for both genders. It is not clear from the paper in what category women drinking between 15, and 30 grams daily fall (Ma, K. et al. 2017). There are other serious issues with this paper, as some of the odds ratios, and numbers of cases and controls differ from the ones in the original papers included in the meta-analysis. (Ji et al. 1996, Shin et al. 2011, Zaridze et al. 2000) Some of the papers, or even journals referenced could not be found for verification of data.

In another meta-analysis of 22 cohort studies no significant association was found between gastric cancer and light or moderate alcohol consumption. Heavy alcohol consumption (<24g/day) was associated with an increased risk for gastric cancer (OR 1.13; 95%CI 1.06-2.21). Female light drinkers <12g/day were at a lower odd for gastric cancer compared to non-drinkers (OR 0.74; 95%CI 0.57-0.98)(He et al. 2017).

A recent pooled analysis of epidemiological studies found no increase in gastric cancer risk in those who consumed up to four alcoholic drinks per day, whereas risk was increased in those who consumed from >4 to 6 drinks/day (OR 1.26; 95% CI, 1.08–1.48) and those who consumed >6 drinks/day (OR 1.48; 95% CI 1.29–1.70). Unlike in the Korean study mentioned above, the increased risk associated with heavy alcohol consumption was similar between *H. Pylori* positive and negative individuals (Rota et al. 2017).

Another meta-analysis of prospective cohort studies on dietary factors associated with risk of gastric cancer showed an association between alcohol overall (OR 1.15; 95%CI, 1.01-1.31), beer (OR 1.21; 95%CI, 1.02-1.43), and liquor (OR 1.22; 95%CI, 1.05-1.43), and gastric cancer. Consumption of wine was not associated (OR 1.02; 95%CI, 0.77-1.34) (Fang, X. et al. 2015).

It is concluded from the above that alcohol consumption increases the risk for upper gastrointestinal tract cancers in a dose-dependent manner. The effect is strongest in oral, pharyngeal and oesophageal cancer, and somewhat lesser in laryngeal cancer, and lowest in gastric cancer.

Gene polymorphisms and upper gastrointestinal tract cancers

Polymorphisms in genes encoding enzymes that are involved in metabolizing alcohol are associated with different risks for upper aerodigestive tract (UADT) cancers. Consumed alcohol is metabolized in the liver by alcohol dehydrogenase (ADH) to acetaldehyde, which is then metabolized by aldehyde dehydrogenase (ALDH) to harmless acetate. Several subtypes of these enzymes exist, but the most studied ones are ADH1B (previously known as ADH2) and ADH1C (ADH3) and ALDH2. Knowledge about the genetic expression and activity of these various enzyme subtypes in upper digestive tract, and salivary gland mucosa is thus far short of drawing the full picture of local acetaldehyde formation.

ADH1B

The *ADH1B*1* allele is the predominant allele in most populations, whereas *ADH1B*2* is associated with an approximately 40 times faster metabolic activity for ethanol *in vitro* (Bosron, Li 1986). This fast allele is commonly found in western and eastern Asian populations, but in under 10-15% of European, African and American populations (Li, H. et al. 2007).

In a recent meta-analysis, it was summarized that the *ADH1B*1/*1* allele is a moderate risk factor for UADT cancers compared to the *ADH1B*2/*2* allele in non-drinkers (ref.) (OR 1.78; 95% CI, 1.02-3.08). Alcohol drinking increases the risk for UADT cancer in the *ADH1B*2/*2* to OR 3.36; 95% CI, 2.65-4.27 compared to ref. Furthermore, the risk is clearly increased in the predominant *ADH1B*1/*1* genotype carrying alcohol drinkers (OR 18.48; 95% CI, 12.95-26.40) compared to non-drinkers with *ADH1B*2/*2* genotype (ref.) (Guo et al. 2012). In conclusion, the faster *in vitro* ADH1B*2 is associated with a decreased cancer risk. It has been suggested that this is due to faster elimination of ethanol from the systemic circulation, or by decreasing overall alcohol consumption(Chang et al. 2012).

No information on the cancer risk associated with the *ADH1B*3* allele, which is found in 25% of the African population, was found in the literature.

ADH1C

Two alleles are known to exist of the *ADH1C* gene. *ADH1C*1* encodes for a fast ADH1C *in vitro*, with an approximately 2.5-fold capacity for ethanol oxidation (Bosron et al. 1993). In a pooled analysis of 7 case-control studies with 1325 cases and 1760 controls there was no risk difference for head and neck cancer observed between *ADH1C *1/*1, *1/*2* and **2/*2* genotypes. (Brennan et al.

2004). In a study focusing on heavy drinkers an increased risk for homozygous genotype *ADH1C*1/*1* was observed for oesophageal, hepatocellular, and head and neck cancer. (OR 2.93; 95%CI, 1.84–4.67, 3.56; 95%CI, 1.33–9.53 and 2.2; 95%CI, 1.11–4.36, respectively) (Homann et al. 2006). A meta-analysis including 23 studies worldwide found slightly lower odds ratios (0.87; 95%CI 0.76 - 0.99) for head and neck cancers in ADH1C*1 homozygotes combined with ADH1C*1/*2 vs. ADH1C*2 homozygotes(Chang et al. 2012).

In Caucasian populations both homozygotic alleles are equally common, with 50-70 percent carrying the heterozygous *ADH1C*1/*2* genotype, whereas in Asians the *ADH1C*2/*2* genotype was rare (Brennan et al. 2004). Salivary acetaldehyde concentration after ethanol ingestion ad libitum was twofold in healthy volunteers homozygous for *ADH1C*1* when compared to subjects with *ADH1C*1/*2* or *ADH1C*2/*2* genotype (Visapaa et al. 2004).

In conclusion, the faster *in vitro* ADH1C*1 is related to higher salivary acetaldehyde concentrations. The overall effect of this genotype seems to be protective of head and neck cancers, but in heavy drinkers there is some evidence pointing to an increased UADT cancer risk possibly due to elevated salivary acetaldehyde concentrations during drinking.

ALDH2

A mutation in the *ALDH2* gene which is found almost exclusively in people of East Asian descent encodes for an inactive ALDH2 enzyme (Harada et al. 1981, Li, H. et al. 2009). The enzyme is inactive in homozygotes, whereas heterozygotes retain 17% of activity in liver tissue (Lai et al. 2014). It was first reported in 1996 that this mutation is linked to an increased risk for oesophageal cancer among alcoholics and heavy drinkers(Yokoyama, A. et al. 1996). On the other hand, the inactive form of this enzyme also protects from alcoholism, due to a ”flushing reaction”, an adverse reaction to even a small amount of ingested alcohol due to accumulation of acetaldehyde. Heterozygotes are capable of heavy drinking, but homozygotes are essentially unable to drink heavily. (Peng et al. 1999). OR for alcohol dependence in carriers of the *ALDH2*2* gene was 0.22; 95%CI 0.18-0.27 in a pooled meta-analysis (Li, D. et al. 2012).

Homozygotes for *ALDH2*2* are practically unable to consume alcohol, and they have a lower risk for oesophageal cancer, (OR = 0.36; 95%CI 0.16-0.80) when compared to *ALDH2*1* homozygotes. However, *ALDH2*1/*2* carried an increased risk for oesophageal cancer, when compared to *ALDH2*1/*1* (OR = 3.19; 95%CI 1.86-5.47). Among heavy drinkers the risk was further increased (OR = 7.07; 95%CI 3.67-13.60), whereas there was no increased risk in non-drinkers (OR= 1.31; 95%CI 0.70-2.47) (Lewis, Smith 2005).

Similar figures were found for heterozygotes in a more recent meta-analysis. The risk for oesophageal cancer in *ALDH2*1/*2* vs. **1/*1* was 1.28; 95%CI 0.91-1.80 vs. 3.12; 95%CI 1.95-5.01 vs. 7.12; 95%CI 4.67-10.86 in never/rare drinkers, moderate drinkers and heavy drinkers respectively. *ALDH2*2/*2* homozygotes had similar risk for oesophageal cancer in never/rare drinkers compared to *ALDH2*1/*1* homozygotes (OR = 1.08; 95%CI 0.48-2.44), whereas an increased risk was observed in moderate and heavy drinkers combined (OR = 4.42; 95%CI 1.72-11.36), it is noted that the authors pooled never/rare drinkers and moderate/heavy drinkers in this analysis due to small number of drinkers in *ALDH2*2/*2* group. (Yang et al. 2010).

Less data is available for oral/oropharyngeal cancer and *ALDH2* genotype, but available data points to an increased risk associated with the inactive *ALDH2*2* allele. In a small case-control study among Japanese alcoholics with 33 cases and 476 controls, risk for oropharyngeal cancer among *ALDH2*1/*2* heterozygotes was greatly increased compared to *ALDH2*1/*1* homozygotes (OR 18.52; 95%CI 7.72-44.44) (Yokoyama, A. et al. 2001). In a Japanese overall population with 147 cases and 92 controls, no significant association between ALDH2 genotype or alcohol drinking and oral cancer was found (Katoh et al. 1999). Another study with, with 114 cases and 33 controls, all who were alcohol drinkers, showed an increased risk for oral cancer in *ALDH2*1/*2* heterozygotes (OR = 2.9; 95%CI 1.1-7.8) (Nomura et al. 2000). In a meta-analysis including six studies, 945 cases, and 2917 controls the OR for head and neck cancer was 0.53; 95%CI 0.28-1 for *ALDH2*2/*2* and 1.83; 95%CI 1.21 – 2.77 for **1/*2* compared to **1/*1* (Boccia et al. 2009).

A case-control study on the interplay of the genetic variants of ALDH2 and ADH1B and oral hygiene in the risk of head and neck cancer showed again an increased risk for head and neck cancer in association with slower ALDH2*1/*2 (OR 1.89; 95%CI 1.36 – 2.62) when compared to normal ALDH2*1/*1. The completely inactive ALDH2*2/*2 was not a risk factor (OR 1.20; 95%CI 0.62 – 2.32). The slow ADH1B*1/*1 was also associated with an increased risk (OR 2.08; 95%CI 1.14-3.80) compared to fast ADH1B*2/*2. The risk was further increased in these subgroups with increased alcohol consumption. In ALDH2 deficient individuals OR was 2.6; 95%CI 1.19-5.75 for moderate drinking (up to 50g/day), and 7.28; 95%CI 2.00-26.74) for heavy drinking. In ADH1B deficients OR for HNC was 2.72; 95%CI 1.43 – 5.17 for heavy drinking (>50g/day)(Tsai et al. 2014).

The risk for stomach cancer was also increased in ALDH2-deficient individuals in a Japanese case control study with 697 cases of stomach cancer and 1372 non-cancer control subjects. OR for stomach cancer in ALDH2-deficients was 1.40; 95%CI 1.11-1.76 in heterozygotes, and 1.73; 95%CI 1.12-2.68 in homozygotes. ALDH2-deficiency combined with heavy drinking

increased the odds ratio to 3.03; 95%CI 1.59-5.79 (Matsuo et al. 2013). Further evidence for the role of acetaldehyde in gastric cancer was provided by a study wherein interaction between heavy alcohol drinking, ALDH2 deficiency, and atrophic gastritis resulted in a substantially increased risk for gastric cancer (OR 3.92; 95%CI 6.4-239) (Yokoyama, A. et al. 2007).

Ethanol metabolism

Absorption and distribution

There is no transport mechanism for ethanol, which is freely absorbed into the water phase of the body. Ethanol is insoluble in fats and oils, although it passes readily through biological membranes through diffusion. The stomach and small intestine are the primary sites for ethanol absorption. Blood flow helps to distribute ethanol into all tissues and fluids, until an equilibrium concentration is achieved which depends on the relative water content of tissues. Ethanol concentrations in blood, saliva, and upper digestive tract contents is essentially equal at equilibrium (Cederbaum 2012, Halsted et al. 1973, Jones 1979).

After ingestion, first pass metabolism of ethanol by the gastric mucosa and liver accounts for a varying amount of ethanol metabolism, decreasing the bioavailability of ethanol when compared to intravenous administration. First pass metabolism increases with delayed gastric emptying (Oneta et al. 1998). Hepatic and gastric first-pass metabolism are not easily distinguished from each other, and the proportion in which they contribute to first pass metabolism of ethanol is unclear. Estimates for contribution of first pass metabolism of ethanol ranges from 1-20% of total ethanol metabolism (Seitz, Poschl 1997).

Metabolism

Ethanol elimination is primarily by metabolism, and only minute amounts are excreted in breath, urine and sweat (~1%) (Holford 1987). The liver is the primary organ for ethanol metabolism, but significant extrahepatic metabolism, up to 40-55%, occurs especially in cirrhosis (Utne, Winkler 1980, Dam et al. 2009). Three distinct pathways for ethanol metabolism are known. In each pathway, ethanol is oxidized to acetaldehyde which is further oxidized to acetate. Acetate can then be converted to acetyl-coenzyme A, which is freely used by the body to produce Co_2, fatty acids, ketone bodies, or cholesterol(Cederbaum 2012).

Alcohol dehydrogenase

Alcohol dehydrogenases catalyse the oxidation of a wide range of alcohols, protecting cells from toxic effects of various alcohols. Six vertebral classes of alcohol dehydrogenase are known to exist (I-VI). ADH:s are cytoplasmic enzymes. Class I (ADH1A, ADH1B, and ADH1C) is the principal alcohol dehydrogenase present in liver tissue, and to lesser extent in other tissues. Class II ADH2 which is also present in liver has a higher K_m for ethanol than Class I ADH1, and thus only participates in higher concentrations of ethanol. Class IV ADH4 is present in the upper aerodigestive tract, and stomach, and probably accounts for the gastric first pass metabolism of ethanol (Jornvall, Hoog 1995, Hoog et al. 2001).

The reversible reaction catalysed by ADH uses NAD/NADH as an electron acceptor / donor. The reaction is limited generally by maximum capacity of ADH, but dissociation of produced NADH, product inhibition by NADH and acetaldehyde, and substrate inhibition by high concentrations of ethanol also limit the reaction (Cederbaum 2012). The physiological substrates for ADHs are not known, and although research has focused on oxidation of ethanol, the ADH reaction strongly favours reduction of acetaldehyde *in vitro*. Thus, in absence of ethanol ADH serves to reduce acetaldehyde concentrations (Deetz et al. 1984). The genetic polymorphism in ADH1B discussed previously not only affects the rate of ethanol oxidation to acetaldehyde, but also the rate of acetaldehyde reduction to ethanol *in vitro* by hepatic ADH1B (Yin et al. 1984).

The K_m for hepatic ADH lies around 0.2mM, and ADH is the principal path for ethanol metabolism in humans (Dam et al. 2009). Different expression patterns of ADH isoenzymes in upper and lower digestive tract may regulate exposure to ethanol metabolites, with high K_m ADH4 being predominant in the upper aerodigestive tract. Mucosal ADH activity contributes little to overall ethanol turnover, but probably plays a part in regulation of local ethanol and acetaldehyde exposure (Yin et al. 1997).

Microsomal ethanol oxidizing system

The microsomal ethanol oxidizing system (MEOS), comprises ethanol oxidation mainly by CYP2E1 (Lieber 1988). CYP2E1 has a K_m of 10 mM for ethanol, and thus becomes of significance at higher concentrations of ethanol. It is inducible 4-10-fold by chronic ethanol intake (Dinis-Oliveira 2016), but there is however no compelling evidence for a significant contribution of MEOS in ethanol metabolism *in vivo* in humans (Dam et al. 2009). The reaction is dependent on NADP and oxygen, and the end products are acetaldehyde, NADPH and water (Dinis-Oliveira 2016). CYP2E1 is also expressed in oral, (Vondracek et al. 2001) oesophageal (Millonig et al. 2011) and gastric mucosa (Kato et al. 2011), although the contribution to local ethanol-acetaldehyde metabolism and exposure is unknown.

Catalase

Peroxisomal catalase can also oxidize ethanol to acetaldehyde in a reaction which requires Hydrogen peroxide (H_2O_2) and produces water. The contribution to total ethanol elimination is regarded as insignificant, due to low rates of H_2O_2 regeneration (Thurman, Handler 1989, Dinis-Oliveira 2016).

Aldehyde dehydrogenase

Acetaldehyde is metabolized to acetate primarily by cytosolic ALDH1B and mitochondrial ALDH2 in a NAD-dependent irreversible reaction. They are expressed in various tissues, with the highest occurrence in liver. (Yoshida et al. 1998). Hepatic ALDH2 is highly active, and only minute amounts, if any acetaldehyde can normally be detected in blood circulation after ethanol ingestion (< 2-5µM, mean peak acetaldehyde) (Nuutinen et al. 1984, Eriksson, Fukunaga 1993, Peng, Yin 2009). However, in ALDH2 deficient individuals with heterozygotic *ALDH2*1/*2* readily measurable amounts have been reported (12-25µM after 0.6g /kg body weight ethanol (Yokoyama, A. et al. 2008).

Figure depicts the main metabolic pathway of ethanol

Extrahepatic ethanol metabolism

As previously discussed, ethanol is mainly metabolized by the liver, but extrahepatic ethanol metabolism plays a part in regulation of local ethanol and acetaldehyde exposure. Measurements of ADH and ALDH activity *in vitro* in surgical tissue samples from various regions of the digestive tract show that in general, ADH-activity is higher than ALDH-activity, expect in pancreas. The ratio of ADH to ALDH activity was 1,8 in stomach, 2,7 in liver, 20,2 in oesophagus. ALDH-activity in tongue and gingiva were too low to be reliably determined (Yin et al. 1993, Dong et al. 1996, Yao et al. 1997). The active class I or II ALDHs are not expressed in gingiva or tongue, whereas ADH4 is (Dong et al. 1996, Yin et al. 1997). Thus, the ability of said tissues to oxidize acetaldehyde is limited, and acetaldehyde accumulates in saliva after alcohol intake (Homann et al. 1997). Local acetaldehyde exposure comes both from "instant" metabolism from the ethanol in a sip of an alcoholic beverage, and "long-term" metabolism from ethanol delivered through the systemic circulation(Linderborg et al. 2011, Helminen et al. 2013).

Microbial ethanol metabolism

Microbes belonging to normal flora in the GI-tract can produce ethanol by anaerobic fermentation through reduction of acetaldehyde by bacterial ADH (Salaspuro, V. et al. 1999). In aerobic conditions, and presence of ethanol, the reaction can flow in the opposite direction, and ethanol is oxidized to form acetaldehyde (Salaspuro, M. 1997). This has been observed in humans in colonic contents in vitro (Tillonen et al. 1998), saliva in vivo (Homann et al. 1997), saliva in vitro (Homann et al. 2000), achlorhydric stomach in vivo (Vakevainen, Tillonen, Salaspuro et al. 2000, Vakevainen et al. 2002) and in vitro (Vakevainen, Tillonen, Blom et al. 2001).

Mouthwashings from patients with oral, pharyngeal or laryngeal cancers when compared to healthy controls, produced more acetaldehyde from incubation with ethanol *in vitro,* indicating that differences in microbial capacity of acetaldehyde production could affect head and neck cancer risk (Jokelainen, Heikkonen et al. 1996). The capability to produce (Jokelainen, Siitonen et al. 1996, Nosova et al. 1998, Vakevainen, Tillonen, Blom et al. 2001) and metabolize (Nosova et al. 1996) acetaldehyde varies considerably between different strains of GI-tract microbes. In 4 out of 5 colonic aerobic bacteria the ADH-activity was higher than ALDH-activity by an order of magnitude (Nosova et al. 1998). In bacterial isolates from gastric juice made achlorhydric by treatment with lansoprazole species of Neisseria and *Rothia,* and *Streptococcus salivarius* were effective acetaldehyde producers, whereas *Stomatococci, Staphylococci* and other *Streptococci* produced less acetaldehyde. ADH activity and acetaldehyde production correlated positively among the strains (Vakevainen, Tillonen, Blom et al. 2001). Oral streptococci and yeast are able to produce significant concentrations of acetaldehyde from ethanol in vitro (Kurkivuori et al. 2006, Nieminen et al. 2009). Several strains of oral streptococci lack ALDH activity altogether, allowing for accumulation of acetaldehyde, although some strains are able to produce acetate from ethanol, indicating ALDH activity (Pavlova et al. 2013). In a study with 65 volunteers, acetaldehyde concentration in mouth air correlated positively and statistically significantly with the amount of tongue coating, but not with other clinical parameters such as presence of *Candida* species, smoking, ALDH2 genotype, or alcohol drinking frequency. The authors suggest that tongue coating acts as a reservoir for acetaldehyde producing microbes. Mechanic removal of tongue coating significantly decreased acetaldehyde concentrations in mouth air (Yokoi et al. 2015). Reduction of acetaldehyde concentration in saliva incubated with alcoholic beverages *in vitro* has been demonstrated using killed *Gluconobacter kondonii* cells, which possess acetaldehyde decomposing properties even in the presence of ethanol (Yamaguchi et al. 2012). This opens up a line of research using microbes in reduction of acetaldehyde exposure. Studies on salivary microbiome metabolism of acetaldehyde *in vivo* have not been published.

Candida Albicans has been shown to produce acetaldehyde from ethanol and glucose when incubated *in vitro* (Tillonen et al. 1999b, Uittamo et al. 2009, Gainza-Cirauqui et al. 2013, Marttila, Uittamo et al. 2013, Marttila, Bowyer et al. 2013). Strains isolated from smokers produced higher concentrations of acetaldehyde, whereas alcohol drinking, current oral squamous cell cancer or oral lichenoid disease had no such effect (Marttila, Uittamo et al. 2013). In one study *Candida albicans* isolates from patients with precancerous oral lesions produced less acetaldehyde from ethanol than strains from healthy controls (Gainza-Cirauqui et al. 2013). *Candida* yeasts have been implicated in the appearance of oral and oesophageal carcinoma, and in a study comparing *Candida* species from patients with oral cancer vs. controls a significant positive association between oral cancer and *Candida* virulence factors, such as ability to form microfilms, and production of hydrolytic enzymes and ability to metabolize ethanol to acetaldehyde was found (Alnuaimi et al. 2016).

The ability of a microbial isolate to produce acetaldehyde *in vitro* does not necessarily correlate with the acetaldehyde production ability when present in the oral microbiome. This was shown with *Neisseria* species, which when isolated are potent acetaldehyde producers, but salivary samples showed an inverse correlation with the relative abundance of *Neisseria* and acetaldehyde production capability of saliva incubated with ethanol *in vitro* (Yokoyama, S. et al. 2018).

In piglets receiving intracolonically administered acetaldehyde, the acetaldehyde was effectively metabolized, as shown by increasing intracolonic acetate and ethanol concentrations (Jokelainen, Matysiak-Budnik et al. 1996). Acetaldehyde is also readily absorbed into the portal blood in rats receiving acetaldehyde into either the colon or stomach. Some of the intracolonically administered acetaldehyde was metabolized to ethanol, and detected in portal venous blood, whereas intragastrically administered was not. It was concluded that acetaldehyde was probably metabolized to ethanol by colonic microbial ADH (Matysiak-Budnik et al. 1996).

Antibiotic treatment with ciprofloxacin was shown to reduce ethanol elimination rate in humans by 9.4% *in vivo*, while concurrently reducing faecal acetaldehyde production, and faecal ADH-activity *in vitro* (Tillonen et al. 1999a). Metronidazole treatment increased intracolonic acetaldehyde concentrations in rats, probably due to replacement of intestinal anaerobes with ADH containing anaerobes (Tillonen et al. 2000). Lactulose reduces ethanol elimination rate, and colonic acetaldehyde concentration in rats after ethanol administration (Zidi et al. 2003). These findings point to a significant contribution of the gastrointestinal microbiome to overall ethanol elimination, and regulation of acetaldehyde concentration in the GI-tract.

Mechanism of carcinogenesis related to ethanol consumption

Ethanol

Alcohol consumption is carcinogenic in humans (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2010). Evidence points to that acetaldehyde is predominantly responsible for carcinogenesis associated with alcohol consumption. Ethanol in itself as a molecule is not capable of producing the necessary mutations required for emergence of cancer (Poschl, Seitz 2004), and there is no significant evidence that ethanol itself is genotoxic (Phillips, Jenkinson 2001). Possible mechanisms for carcinogenesis related to ethanol consumption are summarised below.

Ethanol consumption induces hepatic cytochrome P450 2E1 activity, leading to increased reactive oxygen species, lipid peroxidation and generation of lipid peroxidation products, such as 4-hydroxynonenal which binds to DNA forming carcinogenic exocyclic DNA-adducts. CYP2E1 is also involved in activation of various xenobiotics and procarcinogens, such as aflatoxin. Furthermore, CYP2E1 metabolizes retinol and retinoic acid resulting in cellular hyperregeneration (Seitz, Mueller 2015). Ethanol influences DNA methylation and induces histone modification associated with altered expression of several genes, including oncogenes (Shukla, S. D., Lim 2013). Alcohol may increase permeability of carcinogens into mucosa (Wight, Ogden 1998), increase inflammation, which may promote tumorigenesis (Shukla, P. K. et al. 2016), and decreased immune response which could be associated with cancer progression (Zhang, H., Meadows 2010).

Acetaldehyde

Exposure to acetaldehyde vapor for 52 weeks has been shown to produce laryngeal carcinomas in Syrian golden hamsters (Feron et al. 1982). Furthermore, acetaldehyde exposure by inhalation for up to 28 months caused an increased incidence of carcinomas in nasal mucosa and olfactory epithelium of rats (Woutersen et al. 1986). Acetaldehyde administered in drinking water to rats caused a slight overall increase in malignant tumours (Soffritti et al. 2002). IARC concluded in 1999 that there is sufficient evidence supporting the carcinogenicity of acetaldehyde in animals, but inadequate evidence in humans (IARC 1999). Largely due to epidemiologic evidence from ALDH2-deficient individuals with increased risk for UADT cancers with alcohol consumption, “acetaldehyde associated with the consumption of alcoholic beverages” was classified as carcinogenic in humans in 2009 (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2012).

Unlike ethanol, acetaldehyde probably acts as a direct chemical carcinogen by forming adducts with DNA, which can lead to DNA mutations and consequently cancer. It can also induce sister chromatid exchanges and gross chromosomal aberrations. It can cause structural and functional changes in cellular proteins by binding to them, such as enzymes involved in DNA repair and DNA methylation. (Brooks, Zakhari 2014, Seitz, Stickel 2007). Increased levels of the major acetaldehyde-DNA-adduct N^2-ethylidenedeoxyguanosine can be found in oral mucosal cells after alcohol ingestion, at a level up to 100-fold compared to baseline, and in a dose-responsive manner at 0.3‰, 0.5‰, and 0.7‰ BAC. (Balbo et al. 2012). In this study a similar alcohol administration protocol was used as in these studies on salivary acetaldehyde after ethanol administration, where the target BAC was 0.5‰ (Homann et al. 1997, Linderborg et al. 2011). In the study by Balbo et. al. vodka was diluted by an unspecified amount before administration, unlike in our study where vodka and calvados were sipped neat. However, combining data from these studies makes it is plausible that acetaldehyde reacts with DNA in salivary acetaldehyde concentrations around 100µM.

Increased levels of N^2-ethylidenedeoxyguanosine adducts have been observed in ALDH2-deficient alcohol fed mice liver and stomach, when compared to mice with normal ALDH2 activity (Matsuda et al. 2007, Nagayoshi et al. 2009). Increased amounts of N^2-ethylidenedeoxyguanosine adducts were also observed in white blood cells of alcoholics (Fang, J. L., Vaca 1997). In ALDH2-deficient alcoholics compared with alcoholics with normal ALDH2-activity the levels of three acetaldehyde-derived adducts where significantly elevated (N^2-ethylidenedeoxyguanosine, α-S-, and α-R-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine) (Matsuda et al. 2006). Furthermore, acetaldehyde adducts have been demonstrated in oral tissues of alcohol consuming patients with squamous cell cancer and pre-cancerous oral lesions (Warnakulasuriya et al. 2008). These adducts seem to be a promising marker for acetaldehyde exposure at the DNA-level.

Acetaldehyde has also been shown to cause telomere shortening in various human cell lines. Telomere shortening was observed during exposure to moderate amounts of ethanol (25mM), and acetaldehyde (25µM). Both concentrations are relevant in the upper digestive tract in a social drinking setting. Furthermore, this telomere shortening during ethanol exposure was inhibited by the alchohol dehydrogenase inhibitor 4-methylpyrazole (Harpaz et al. 2018).

Acetaldehyde has been found to be able to form mutagenic Cr-PdG adducts *in vitro* in the presence of an abundance of spermidine at acetaldehyde concentrations of 100µM and above (Theruvathu et al. 2005). It can inhibit O^6-methylguanine transferase, a DNA-repair enzyme, at concentrations as low as 0.01µM (Espina et al. 1988). It is however unknown how these *in vitro*

findings translate to *in vivo* situations, and thus any estimates on what level of acetaldehyde concentrations are required for carcinogenesis in humans, and whether there is any safe lower limit for acetaldehyde exposure, are tenuous at best (Balbo, Brooks 2015).

Human *ALDH2* "knock-out model" for acetaldehyde exposure in the upper gastrointestinal tract.

Combining epidemiological and biochemical studies focusing on the point mutation in the *ALDH2* gene provides evidence proving the association between local acetaldehyde exposure, and upper digestive tract cancer (Lachenmeier, Salaspuro 2017, Nieminen, Salaspuro 2018). The two main findings leading to this conclusion are that ALDH2-deficient individuals have approximately twice as much acetaldehyde in their saliva after a dose of alcohol when compared to ALDH2-actives. The acetaldehyde exposure endures for as long as there is alcohol present in the body (Vakevainen, Tillonen, Agarwal et al. 2000, Vakevainen, Tillonen, and Salaspuro 2001, Yokoyama, A. et al. 2008, Maejima et al. 2015, Yokoyama, A. et al. 2016). Secondly, alcohol consumption combined with ALDH2-deficiency associates with a significantly increased risk for upper gastrointestinal tract cancer (Yokoyama, A. et al. 1996, Nomura et al. 2000, Lewis, Smith 2005, Boccia et al. 2009, Matsuo et al. 2013).

The benefit of this model is that nature has randomized millions of individuals to different amounts of acetaldehyde exposure, and this also allows for minimizing the effect of confounding factors such as smoking, diet, oral hygiene, HPV, different beverages, drinking habits and BMI (Lachenmeier, Salaspuro 2017).

This model, although comprised of a combination of data from several studies, has allowed for the estimation of the amount of increased acetaldehyde exposure needed to cause an increased risk for UADT cancer. By multiplying salivation rate by salivary acetaldehyde concentration during drinking and duration of acetaldehyde exposure Lachenmeier and Salaspuro calculated that in ALDH2 deficients drinking heavily (7 drinks, 77g/day) an increased acetaldehyde exposure of 6.7 μg/kg bw/ day is achieved. This level of alcohol consumption is associated in ALDH2 deficients with a OR of 7.28 for head and neck cancer, and 7.12 for oesophageal cancer (Lachenmeier, Salaspuro 2017). This amount of acetaldehyde in a person weighing 80kg amounts to 536 μg / day or 12 μmol / day. A seemingly low amount. In comparison, according to our data a single shot (4 cl) of calvados (1780 μmol/l) contains 71 μmol of acetaldehyde (Linderborg et al. 2008). The problem with this model is that it is based on the flow of saliva, and acetaldehyde concentration in it, and attempts to calculate a total dose of acetaldehyde,

when in truth acetaldehyde is produced continuously. This issue is addressed in another model as described below.

Nieminen and Salaspuro proposed another model for estimating increased acetaldehyde exposure in ALDH2 deficients, and combining it to epidemiological data on cancer risk. In this model the average increased acetaldehyde concentration in ALDH2 deficients is multiplied by the estimated time of exposure / day in order to receive an area under the curve of exposure time multiplied by concentration. Exposure to salivary acetaldehyde from cigarette smoking is also calculated, and the AUC:s for calculated exposures of each source for acetaldehyde correspond nicely to odds ratios for oropharyngeal cancers obtained from epidemiological data (Nieminen, Salaspuro 2018). The model is useful when comparing exposure to acetaldehyde, when salivary acetaldehyde concentration is known, and correlating it to risk data from epidemiological studies. However, the paper also suggests extrapolation to yoghurts and fruits with high acetaldehyde concentration, but low or no ethanol concentration. These extrapolations should be considered carefully, as there is currently no data on salivary concentration of acetaldehyde after ingestion of foodstuffs or beverages containing acetaldehyde without ethanol. Yoghurt and fruits usually contains substantial amounts of acetaldehyde (Uebelacker, Lachenmeier 2011), yet consumption of yoghurt was associated with a decreased risk of UADT cancer in a Japanese case-control study of 959 patients with UADT cancer and 2877 controls (Kawakita et al. 2012).

The importance of acetaldehyde and ALDH2-activity in carcinogenesis was further underlined in a study on mice, and cultured human oesophageal keratinocytes. It was shown that alcohol drinking in mice *in vivo*, and acetaldehyde exposure in cell cultures promoted ALDH2 expression. Furthermore, N^2-ethylidenedeoxyguanosine concentration in mouse and human keratinocytes, representing acetaldehyde-derived DNA damage was higher in ALDH2 knockout-mice than in controls, and further increased by treatment with acetaldehyde. Finally, forced ALDH2-production decreased N^2-ethylidenedeoxyguanosine levels. This evidence adds to the importance of ALDH2 in oesophageal tissue in protection against DNA damage caused by acetaldehyde (Amanuma et al. 2015).

Acetaldehyde exposure

From ethanol metabolism

There is 11.71 grams of ethanol in a typical bottle of beer (0.33l, 4.5 vol%) This ethanol, when metabolized produces 11.25g of acetaldehyde. The bulk of this metabolism takes place in the liver, where acetaldehyde is immediately

oxidized to acetic acid by hepatic ALDH enzymes (Nuutinen et al. 1984). The elimination of acetaldehyde in the oral cavity is far from as effective as in liver, and microbial metabolism of ethanol leads to accumulation of marked amounts of acetaldehyde in saliva after ingestion of moderate amounts of ethanol (Homann et al. 1997). Poor dental status leads to increased acetaldehyde production from ethanol in saliva (Homann et al. 2001). Acetaldehyde exposure is further increased by acetaldehyde formed instantly after a sip of an alcoholic beverage (Linderborg et al. 2011). Significant concentrations of acetaldehyde were also found in saliva immediately after using alcohol containing mouthwashes (Lachenmeier, Gumbel-Mako et al. 2009, Moazzez et al. 2011).

Ethanol is not only found in alcoholic beverages, but also in varying amounts in foods. Ethanol can stem from adding an alcoholic beverage during preparation, or from fermentation of the food, or an ingredient thereof. In a German survey the researchers found ethanol concentrations up to 2.6 g/L in vinegar, 2.15 g/L in malt beer, and 0.86 g/L in grape juice. Some types of bread also contained up to 1.28 g / 100g ethanol, while on average breads contained 0.22g / 100g ethanol. The amounts are relatively small when compared to ethanol in alcoholic beverages (Gorgus et al. 2016). The amount of ethanol remaining in a dish after an alcoholic beverage is added during preparation depends mostly on for how long the dish is being cooked, and how freely ethanol is allowed to evaporate. The amount of ethanol in a single serving of food is however approximately only a tenth to a fifth of the amount of ethanol in a single serving of an alcoholic beverage (Augustin et al. 1992). Whether ethanol in food contributes to acetaldehyde exposure and cancer risk is unknown. As discussed previously, there is no safe lower limit of alcohol consumption in relation to overall cancer risk. Even light drinking is a risk factor for oropharyngeal and oesophageal cancer (Bagnardi et al. 2013), whereas light drinking does not seem to increase the risk of pharyngeal or gastric cancer. There is clear evidence of dose dependency with regard to alcohol consumption and upper digestive tract cancer, and thus it seems unlikely that the small amounts of alcohol in foods contributes significantly to cancer risk, but this is something that has yet to be investigated. It can be expected that acetaldehyde related carcinogenicity from foods produced by fermentation that contain some alcohol (0-5%) is of the same order of magnitude as from light drinking. There might exist an epidemiological bias, due to that there is no systematic data on the use of foods with ethanol or acetaldehyde content and upper digestive tract cancer risk. Nor is there systematic information available on acetaldehyde content in foods.

Outside ethanol metabolism

Acetaldehyde is formed in small amounts through enzymatic breakdown of threonine, by threonine aldolase (Lin, Greenberg 1954). A small amount of

acetaldehyde can be produced by bacterial metabolism without ethanol drinking, but not in measurable amounts in blood or saliva (Vakevainen, Tillonen, Agarwal et al. 2000, Vakevainen, Tillonen, and Salaspuro 2001, Yokoyama, A. et al. 2008). The bulk of acetaldehyde exposure is through alcohol drinking by metabolism of ethanol to acetaldehyde.

Acetaldehyde can also be found in varying amounts in different alcoholic beverages (Lachenmeier, Sohnius 2008, Boffetta et al. 2011, Paiano et al. 2014) and in some foodstuffs, especially fruits and yoghurts (Uebelacker, Lachenmeier 2011, Miyake, Shibamoto 1993). Acetaldehyde is a fruit metabolite, and a natural aroma component, that accumulates during fruit ripening (Pesis 2005). Because of its aromatic properties, and insufficient data on carcinogenicity until recent times it is still used widely as a food additive (Feron et al. 1991).

Tobacco smoke contains acetaldehyde among other toxic constituents, and it is readily absorbed readily in the mouths of smokers (Dalhamn et al. 1968). Salivary acetaldehyde concentration increased rapidly from zero to 261µM during smoking a single cigarette, and decreased to baseline in no more than 5 minutes after cessation of smoking (Salaspuro, V., Salaspuro 2004). Smoking also seems to alter acetaldehyde metabolism in the mouth and saliva. Smokers had approximately twofold salivary acetaldehyde concentrations after ethanol ingestion without concurrent cigarette smoking compared to non-smokers. Acetaldehyde production in saliva from ethanol *in vitro* was also higher in smokers vs. non-smokers (Homann et al. 2000).

Acetaldehyde exposure from foods has been estimated to be from 3mg to 200mg / day for a person weighing 75kg (Uebelacker, Lachenmeier 2011). No studies have so far been undertaken to determine how this acetaldehyde contributes to mucosal acetaldehyde exposure.

Acetaldehyde in alcoholic beverages is primarily formed from sugar as a by-product of alcoholic fermentation by yeasts. The rate of acetaldehyde production during fermentation is influenced by a multitude of factors, including species of yeast, temperature, presence of oxygen, and sulphur dioxide concentration. Sulphites are also used as additives in winemaking for their antimicrobial, antioxidative and acetaldehyde binding properties. Sulphites have a strong affinity for binding to acetaldehyde, reducing concentrations of free acetaldehyde in wines (Liu, Pilone 2000). In distilled alcoholic beverages the acetaldehyde concentration depends largely on the method of distillation, but also maturation of beverages (Nykänen, Suomalainen 1983, pp. 52-53).

Acetaldehyde concentrations in saliva after drinking different alcoholic beverages with different concentrations of acetaldehyde have been studied by

Yokoyama et. al. and Lachenmeier et. al. Yokoyama measured salivary acetaldehyde immediately after drinking, and at 30-minute intervals, and no difference in salivary acetaldehyde concentration was observed apart from the first sample, where acetaldehyde concentrations were significantly higher after drinking diluted calvados or shochu, containing 600μM acetaldehyde, compared to wine containing 250μM acetaldehyde. The acetaldehyde concentration in beverage had no effect on blood acetaldehyde concentration (Yokoyama, A. et al. 2008). In the study by Lachenmeier no simultaneous alcohol ingestion was studied. The results were in line with previous data. They found that acetaldehyde in beverages affect the salivary acetaldehyde concentration for less than two minutes after tasting a beverage for 30 seconds (Lachenmeier, Monakhova 2011).

Cysteine and acetaldehyde

The semi-essential amino acid L-cysteine condenses readily in a nonenzymatic, reversible reaction with acetaldehyde in physiological conditions forming 2-methyl-4-thiazolidine-carboxylic acid (MTCA) (Reischl et al. 2012). Thus, it could potentially be used as harm reduction by reducing exposure to acetaldehyde. L-cysteine has been shown to bind acetaldehyde derived from tobacco smoke *in vitro* (Braven et al. 1967), and in saliva *in vivo* (Salaspuro, V. J. et al. 2006), and decrease acetaldehyde concentration in saliva after alcohol drinking (Salaspuro, V. et al. 2002). A buccal tablet releasing cysteine and chlorhexidine was also effective in reducing acetaldehyde concentration in saliva after ethanol exposure *in vivo* (Juliano et al. 2011). The safety of cysteine supplementation is examined next.

Safety of cysteine

L-Cysteine is an endogenous excitotoxin that can damage the central nervous system in experimental animals with immature central nervous systems before the development of an intact blood-brain barrier (Olney et al. 1990). An excess cysteine supplementation of 7.5 times the dietary requirement was lethal in chicks after 5 days of treatment, the cause of death being severe metabolic acidosis. An excess cysteine supplementation in food decreased weight gain in pigs (Dilger et al. 2007, Dilger, Baker 2008).

Evidence for toxicity in humans is however scarce. Kartal-Hodzic et. al. examined the toxicity and permeability of cysteine and MTCA on Caco-2 cells and found no evidence of harm to these cell cultures at concentrations up to 1200 μg/ml for L-cysteine and 600 μg/ml for MTCA (Kartal-Hodzic et al. 2013). Ingestion of a single dose of 5g and 10g of cysteine produced nausea and light-headedness in healthy subjects (Carlson et al. 1989). The liver is an

effective regulator of cysteine. By synthesizing glutathione, which serves as a cysteine reservoir it keeps cysteine levels appropriate, allowing for normal metabolism, but keeping cysteine levels below the threshold for toxicity (Stipanuk et al. 2006).

Cysteine is found in proteins in normal diet, and it is also taken as dietary supplements. It is used as an additive in flour to improve baking properties. Daily intake requirements in humans according to WHO for sulphur containing amino acids cysteine and methionine combined is 13mg/kg body weight (Energy and protein requirements report of a Joint FAO/WHO/UNU Expert Consultation. 1985). The mean daily intake of cysteine according to data from a survey in Americans was 1g/day in all age groups, whereas the highest intake of 2.2g/day was found at the 99th percentile of men at 51-70 years of age. It has been concluded that there is insufficient data to establish a safe upper limit for cysteine intake from supplements (Panel on Macronutrients et al. 2005). Some research points to a need for cysteine supplementation, and positive effects thereof, in elderly people (Droge 2005, Nimni et al. 2007).

Safety of MTCA

What happens to MTCA in the digestive tract is largely unknown, as are the potential effects of MTCA on humans in general as the subject is insufficiently studied. MTCA can break down spontaneously into cysteine and acetaldehyde. Radioactively labelled ^{14}C-MTCA, fabricated from cysteine and ^{14}C-acetaldehyde, was given to rats intraperitoneally. 52.8% of the radioactive dose was expired as $^{14}CO_2$ within the first 4 hours, indicating rapid dissociation of acetaldehyde and subsequent metabolism (Nagasawa et al. 1982). In a similar experiment 13.6% of the radioactivity was recovered in urine within 4 days, also suggesting active metabolism of MTCA. The finding was limited by the fact that faecal excretion of MTCA was not assessed (Kallama, Hemminki 1983).

MTCA can be freely nitrosated in a nonenzymatic reaction to NMTCA in presence of nitrite, optimally in acidic conditions (pH4.5). Thus, NMTCA can form spontaneously from acetaldehyde, cysteine and nitrite. NMTCA when given orally to rats, is recovered up to 95% in urine and faeces within 2 days from administration (Ohshima et al. 1984). NMTCA can be detected in human urine, albeit in very small amounts of 0.4 -27.6 µg / 24h. The urine of cigarette smokers contained twice as much NMTCA as non-smokers, possibly due to endogenously derived NMTCA from acetaldehyde in cigarette smoke (Tsuda et al. 1987).

MTCA can be detected in human blood after consumption of ethanol, suggesting that it is endogenously formed from cysteine and acetaldehyde, inactivating endogenous or exogenous acetaldehyde. Peak blood MTCA concentration was 12.6mg/L (85µM) 4 hours after an ethanol intake of 0,5g /kg bw (Reischl et al. 2012).

In conclusion, there is evidence that cysteine can be used to reduce acetaldehyde concentrations in saliva. The safety of cysteine supplementation is however not known, and a safe upper limit for cysteine supplementation has not been established.

Atrophic gastritis and gastric achlorhydria

Helicobacter pylori infection is a significant risk factor for developing chronic atrophic achlorhydric gastritis and subsequent development of gastric cancer (Kuipers et al. 1995, Correa et al. 1990). The risk ratio for gastric cancer in people chronically infected with *H. Pylori* seems to be around 2-3-fold (Danesh 1999). Severe atrophic gastritis has been known to be a risk factor for gastric cancer for over 30 years (Sipponen et al. 1985). In a large Finnish study *H. pylori* infection was associated with a 5.8-fold risk for stomach cancer (95% CI 2.7-15.3), and serologically confirmed atrophic gastritis was associated with a 9.1-fold risk for stomach cancer (95% CI 2.9-30) (Vohlonen et al. 2016). Pernicious anaemia has also been linked with an approximately threefold risk of stomach cancer in epidemiologic studies. Again, this is probably through the underlying atrophic gastritis, and achlorhydria (Brinton et al. 1989). In a Japanese case-control study with 45 cases of gastric carcinoma ALDH2 deficiency, and chronic atrophic gastritis were independent risk factors for gastric cancer, and the combination of severe chronic atrophic gastritis and ALDH2 deficiency resulted in a 39-fold risk for development of gastric cancer (OR=39.2, 95%CI 6.4-239) (Yokoyama, A. et al. 2007).

Interestingly also the risk for oesophageal squamous cell carcinoma (ESCC), but not adenocarcinoma was increased threefold in atrophic gastritis related to pernicious anaemia (Ye, Nyren 2003). In another Japanese case-control study with 63 cases of ESCC, the investigators examined the association of hypochlorhydria, and ALDH2-deficiency with ESCC and found an increased risk for ESCC in hypochlorhydria alone (OR=4.4, 95%CI 1.3-15.0). No increased risk was observed in ALDH2 deficiency alone (OR=1.6, 95% CI 0.5-5.2), whereas the combination of inactive ALDH2 and hypochlorhydria resulted in a markedly increased risk for ESCC (OR=21.8, 95%CI 4.9-97.0) (Oikawa et al. 2010).

Atrophic gastritis leads to increased bacterial growth in the stomach through achlorhydria. At gastric pH less than 4.0 99.9% of bacteria are killed

in less than 30 minutes (Giannella et al. 1972). This gastric acid barrier is effective against most bacteria with the exception of *Helicobacter Pylori* and some acid resistant lactobacilli (Marshall et al. 1990, Aiba et al. 2015). In achlorhydria the stomach is colonized with a large number of bacteria (Drasar et al. 1969). Gastric bacterial counts are also increased by acid suppressive drugs such as omeprazole(Verdu et al. 1994), and cimetidine (Ruddell et al. 1980).

Helicobacter pylori also possesses ADH activity, and is able to produce significant concentrations of acetaldehyde when incubated in clinically relevant ethanol concentrations *in vitro* (Roine et al. 1992).

In healthy volunteers who served as their own controls, Lansoprazole 30mg twice a day for 7 days resulted in decreased pH of gastric juice, increased bacterial count in gastric juice, and in an increased gastric juice acetaldehyde concentration (mean ± SEM 55.4 ± 8.0 μM vs. 22.1 ± 2.3 μM) after ethanol ingestion of 0.6g/kg body weight (Vakevainen, Tillonen, Salaspuro et al. 2000). Similar results were obtained when comparing volunteers with atrophic gastritis to healthy volunteers. Gastric juice acetaldehyde concentrations were (mean ± SEM) 44.5 ± 9.2 μM vs 9.8 ± 0.9 μM at 30 minutes after instillation of ethanol 0.3g/kg bw via nasogastric tube (Vakevainen et al. 2002).

In conclusion, achlorhydria results in increased gastric juice acetaldehyde concentration after ethanol ingestion. ALDH2-deficient healthy volunteers had approximately 5 times higher peak gastric juice acetaldehyde concentrations after intragastric ethanol infusion, when compared to ALDH2-proficient peers (Maejima et al. 2015). An increased acetaldehyde exposure could explain the increased risk for gastric cancer in ALDH2-deficient individuals with severe chronic atrophic gastritis, as discussed above in this chapter.

AIMS OF THE STUDY

Acetaldehyde associated with consumption of alcoholic beverages is carcinogenic in humans. The bulk of acetaldehyde exposure comes from ethanol metabolism but acetaldehyde in alcoholic beverages might also have significance, especially regarding different risks of upper aerodigestive tract cancers associated with consumption of different beverages, in this case calvados.

Furthermore, a feasibility study on reducing exposure to acetaldehyde in achlorhydric stomach with L-cysteine was conducted.

Specific aims were as follows:

I. To investigate acetaldehyde concentrations in calvados from industrial and rural sources and compare them to other alcoholic beverages. The findings could elucidate the role of acetaldehyde found in alcoholic beverages in the increased risk for oesophageal cancer connected to consumption of calvados.
II. To examine acetaldehyde concentrations in saliva after single sips of strong alcoholic beverages containing either no acetaldehyde or high concentrations of acetaldehyde, and thus compare the resultant exposure to acetaldehyde from these beverages, and to explore the significance of acetaldehyde in beverages to overall acetaldehyde exposure in the mouth.
III. To determine if L-cysteine can be used to bind acetaldehyde produced in the achlorhydric stomach from ingested ethanol and reduce the exposure to acetaldehyde.

MATERIALS AND METHODS

Potential mechanism for calvados-related oesophageal cancer

Samples

Eighteen samples of farm-made calvados were collected in the Manche, Orne, Eure and Seine-inferieure areas in Normandy in 2001-2002. Two samples of farm-made cognac were obtained in Gironde of southwestern France. The samples were collected with disposable syringes and needles and stored in vacutainer tubes at 6°C until analysis. Samples of commercially available beverages were purchased from Alko Inc, and samples were extracted just prior to analysis from previously unopened bottles. These included seven different factory-made calvadoses, four cognacs, two scotch blended whiskies, two rums, one pear-cognac liqueur, one vodka, three white wines, three red wines, three beers and three ciders. These were grouped as 25 calvadoses, 12 other spirits, 6 wines and 6 beers and ciders. Every commercially available calvados from Alko was obtained, whereas other beverages were selected by availability in miniature bottles.

Analysis

Samples were diluted for analysis, based on estimated ethanol concentration, in purified water. Spirits were diluted 1:100, wines 1:30 and beer and cider 1:10. 500µl of the diluted sample was transferred into a glass vial, and subsequently analysed by headspace gas chromatography (Perkin Elmer Autosystem gas chromatograph, Massachusetts, USA). For acetaldehyde analysis, the vials were heated to 37°C. The parameters for gas chromatography were as follows: Column 60/80 Carbopack B/5% Carbowax 20 M, 2m x 1/8″; oven temperature 85°C; transfer line and detector temperature 200°C; carrier gas flow rate (N_2) 20ml/min. For ethanol analysis, the vials were further diluted 1:10, and the headspace vials were heated to 65°C. Gas chromatograph parameters were as above. Statistical analysis was performed with SPSS 13.0.1. (SPSS Inc, Chicago, IL, USA) using one-way ANOVA and Tukey's HSD.

A single sip of a strong alcoholic beverage causes exposure to carcinogenic concentrations of acetaldehyde in the oral cavity

Subjects

Eight healthy volunteers aged 26 ± 4 (years ± SD) participated in the study. There were 4 females, and 4 males. Mean body weight ± SD was 78 ± 18 kg. The volunteers were non-smokers and social drinkers consuming less than 14/20 (female / male) doses of alcohol per week. None of the volunteers had received any antibiotics for 1 month preceding the study, and they were asked to refrain from alcohol intake for 24h prior to the study.

Beverages

Calvados containing 2400μM acetaldehyde or 96% ethanol diluted in water were used. Final ethanol concentration in each beverage was 40 vol. %. The ethanol beverage contained no acetaldehyde. Ethanol and acetaldehyde concentrations of the beverages were measured after 1:100 dilution by headspace gas chromatography as described above.

Study design

The ethics committee of the department of medicine, Helsinki University Central Hospital approved the study, and informed consent of the participants were obtained. The subjects served as their own controls.

A light breakfast was allowed at least 90 minutes prior to commencement of the experiment. To simulate a sip of alcoholic beverage the volunteers were given 5ml of each beverage to taste in their mouths for 5 seconds, whereafter they spit several times to expunge all beverage. Samples were collected by spitting at 30 seconds, 2, 5 and 10 minutes. A fifteen-minute wash-out period was used between beverages. The order in which the beverages were tested was varied between participants.

A test run using 40% ethanol was performed 25 minutes prior to the actual test in order to accustom the participants to the protocol, and to minimise the possible difference in subsequent measurements due to previously taken alcohol.

To examine the effect of alcohol in the bloodstream the tests were repeated at a blood alcohol content of 0.4-0.5 ‰. Participants consumed 0.5g / kg body weight ethanol diluted in water to 10 vol%. Blood alcohol concentration was monitored by a breath alcohol content analyser (Dräger Alcotest 7410 Plus,

Lubeck, Germany) at 5-minute intervals until it was stabilized at 0.4-0.5 ‰. A control salivary sample was then taken, and the sampling run was repeated for the first beverage. Then the volunteers received an additional dose of ethanol (0.05g / kg body weight), whereafter the blood alcohol content was again monitored until stable at 0.4 – 0.5 ‰ and the sampling run was repeated for the other beverage.

Acetaldehyde and ethanol analysis

450µl of saliva was transferred into headspace vials containing 50µl of 6M perchloric acid. Acetaldehyde and ethanol concentrations were analysed by headspace gas chromatography as described in the previous section. The ranges of detection for acetaldehyde and ethanol were 2.5-800µM and 1-150mM respectively.

Statistical analysis was done using SPSS 15.0.1 statistical software (SPSS Inc., Chicago, IL, USA). Wilcoxon's nonparametric test for paired samples was used to test for statistical significance.

Reducing carcinogenic acetaldehyde exposure in the achlorhydric stomach with cysteine

Preparation of cysteine and placebo capsules

The capsules, prepared at the University Pharmacy in Helsinki, contained 50 mg of l-cysteine as an active ingredient. Five hundred grams of l-cysteine (Gonmisol S.A., Barcelona, Spain), 500 g of Eudragit RS-PO forming the matrix structure (Evonik Rohm GmbH, Damstadt, Germany), and 1 kg of calcium hydrogen phosphate as an inactive additive ($CaHPO_4$, Emcompress® Anhydrous; Mendell a Penwest Company, Lakeville, MN) were mixed in a Turbula Powder Blender (Glen Mills Inc., Clifton, NJ) for 10 minutes, and the mixture was wet-granulated using ethanol. The wet granules were sieved using a 2-mm sieve and thereafter allowed to dry at room temperature in a fume hood for 24 hours. The dried granules were sieved using 1.68 mm and 1.18 mm sieves, and the fraction between 1.68 mm and 1.18 mm was collected for capsulation. Simultaneously, a placebo formulation where l-cysteine was replaced with the same amount of $CaHPO_4$ was prepared following exactly the same method. The matrix granules formed were weighed into hard gelatine capsules to ease the administration such that each capsule contained
200 mg of granules, equalling 50 mg of l-cysteine. The l-cysteine concentration of the granules was determined using a capillary method (400 mg of granules contained 98 mg of l-cysteine).

Dissolution Test for the Capsules

Dissolution tests for the capsules were performed according to the USP I method (USP 24) (The United States Pharmacopeia 2001). A standard curve was prepared between 0.01 and 0.6 mg/ml (y = 2.196 + 0.0016, r2 = 0.9999). The medium used was 500 ml of pH 1.2 HCl buffer. The rotation rate of the baskets was 100 rpm, and the temperature of the medium was +37°C (±0.5). Samples were taken at 5-minute intervals for the first half hour and thereafter at 10-minute intervals for the remaining 2 hours. L-cysteine was detected in flow-through cells (10 mm) at a wavelength of 213 nm. The results were calculated by using dissolution software. The system was equipped with a bath and pump (Sotax AT7 UV Dissolution System; Sotax, Allschwil, Switzerland) and a spectrophotometer (Perkin Elmer, Lambda 25; PerkinElmer, Inc., Waltham, MA); the software used for the test and for calculating the results was WinSotax (Sotax).

Subjects

Seven subjects with atrophic gastritis (5 women, 2 men) were enrolled. Mean age ± SD was 57 ± 7 years and mean body weight 75 ± 22 kg. The mean serum gastrin level of the subjects was 417 pM (range 192 to 968 pM, upper normal limit 50 pM). Pepsinogen-1 level was below the detection threshold of 25 µg/l in all volunteers. A routine follow-up gastroscopy with biopsies had been performed on each participant within 1 year prior to the study, and chronic atrophic corpus gastritis without concurrent *H. pylori* infection had been confirmed histologically in all subjects. All volunteers were non-smokers and normal social drinkers, with an average consumption of 50 g or less of ethanol per week. Five of the subjects were receiving vitamin B12 substitution, 1 had medication for hypertension and hypercholesterolemia, and 2 had medication for hypothyroidism; otherwise, the volunteers were clinically healthy. None of the volunteers had received any antibiotics or medication that influences the acidity of the stomach for 1 month preceding the study.

Study Design

The study was approved by the ethics committee of the department of medicine at the Helsinki University Central Hospital, and also by the Finnish National Agency for Medicines. Informed consent was obtained.

The study was randomized, double-blinded and placebo controlled. Each participant served as their own control. The two study days were separated by at least three days. The volunteers were admitted to the department of gastroenterology at Helsinki University Central Hospital, and all studies started between 8 and 10 AM. Volunteers were told to refrain from alcohol

intake for 24 hours prior, and food intake for 12 hours prior. Subjects were told to report any possible side effects during and after the experiments.

Nasogastric intubation to a depth of 55cm (Duodenal tube Levin, CH10; Unomedical, Birkeroed, Denmark) was performed using Xylocain gel lubrication (Astrazeneca, Södertälje, Sweden) Participants were given 100ml water to facilitate swallowing of the tube, and location of tube was confirmed by aspiration of gastric contents. Participants laid on their left sides for the duration of the test to delay gastric emptying. Four capsules containing either 200mg cysteine in total, or placebo were given double blindly, with 200ml water. Ethanol (0.3g/kg body weight) diluted in water to 15 vol% was infused through the nasogastric tube. Samples of gastric juice (5ml) were aspirated through the tube at 5-minute intervals up to 60 minutes after the ethanol infusion, or until aspiration was no longer successful, indicating stomach emptying. Samples were analysed for pH, acetaldehyde, ethanol, and cysteine concentrations. We measured pH using a glass electrode and a digital pH meter (WTW pH-521, Weilheim, Germany)

Acetaldehyde concentration was analysed by headspace gas chromatography, as described above in the previous original publications in this thesis. 450µl of sample was added to 50µl 6M perchloric acid for acetaldehyde analysis. For ethanol analysis, the sample was diluted 1:10 in purified water and 500µl was transferred into headspace vials for analysis. Duplicate samples were analysed, and the means were used in statistical analysis.

L-Cysteine analysis of gastric juice samples.

L-cysteine concentration of gastric juice was measured by HPLC. Two parallel samples were prepared and analysed. 60 µl of gastric juice was measured into a test tube, and 30 µl of pH 7.4 phosphate-buffered saline solution (Ph.Eur.) and 30 µl of 20 vol% Tri-n-butylphosphine in dimethylformamide were added. The samples were incubated for 30 minutes at +4°C, after which 90 µl of cold 10% trichloroacetic acid containing 1 mM Na2EDTA was added, and the samples were vortexed for 2 minutes and then centrifuged (10 minutes, 2,490·g). Fifty microliters of supernatant were pipetted into a test tube containing 125 µl of pH 9.5 borate buffer with 4 mM Na2EDTA, 10 µl of 1.55 M sodium hydroxide, and 50 µl of 2 mg / ml 4-Fluoro-7-Sulfobenzofurazan, Ammonium salt (SBD-F) solution in borate buffer. The samples were incubated for 60 minutes at +60°C so that a yellow derivate was formed. Thereafter, 150 µl of the solution was pipetted into HPLC inserts. Injection volume was 10 µl. The system was equipped with a Waters Model 501 piston pump (Waters, Milford, MA), a Waters 717 Auto-sampler, a Waters 484 tuneable absorbance detector, and a Millennium 32 Chromatography Manager workstation. The isocratic mobile phase was pH 7.0 phosphate buffer

and methanol (95:5). The flow rate was 1 ml / min and retention time was 6 minutes. l-cysteine concentration was determined using a fluorescence detector (excitation 385 nm, emission 515 nm).

Statistical analysis

Statistical significance was tested by Wilcoxon's nonparametric test for paired samples. Correlations were tested using Spearman's rho. A p value less than 0.05 was considered statistically significant. Statistical analysis was carried out using SPSS statistical software (SPSS 15.0.1; SPSS Inc, Chicago, IL, USA)

RESULTS

Potential mechanism for calvados-related oesophageal cancer

We found significantly higher mean acetaldehyde concentrations in calvados (1780 ± 861 µM, mean ± SEM; (range 451–3928 µM); n = 25) compared to other spirits (1251 ± 1155 µM (range 0–4339 µM), $p < 0.05$), wines (275 ± 236 µM (range 0–536 µM), and beer and cider (233 ± 281 µM (range 0–734 µM), $p < 0.001$) (fig. 1). The highest acetaldehyde concentration of 4339 µM was found in a sample of farm-made cognac.

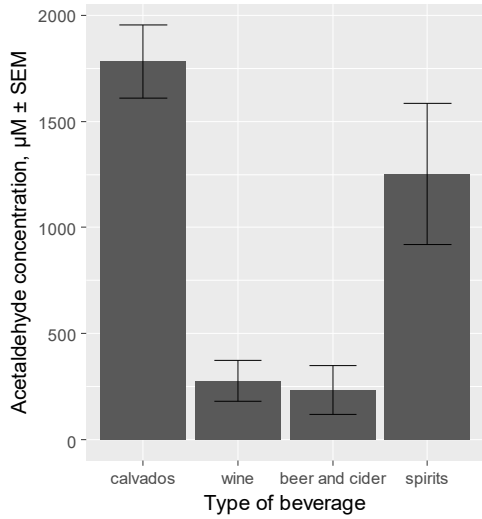

Fig.1 Acetaldehyde concentrations on groups of beverages. The calvados and other spirits groups differ significantly from each other ($p < 0.05$), as well as from the wine, and beer and cider groups ($p < 0.05$).

There was a positive correlation between ethanol concentration and acetaldehyde concentration among all beverages tested ($\rho = 0.748$, $p < 0.001$; n=49), that persisted also when excluding wines, beers, and ciders ($\rho = 0.382$, $p < 0.05$; n=37) This is demonstrated in a scatter plot (fig.2).

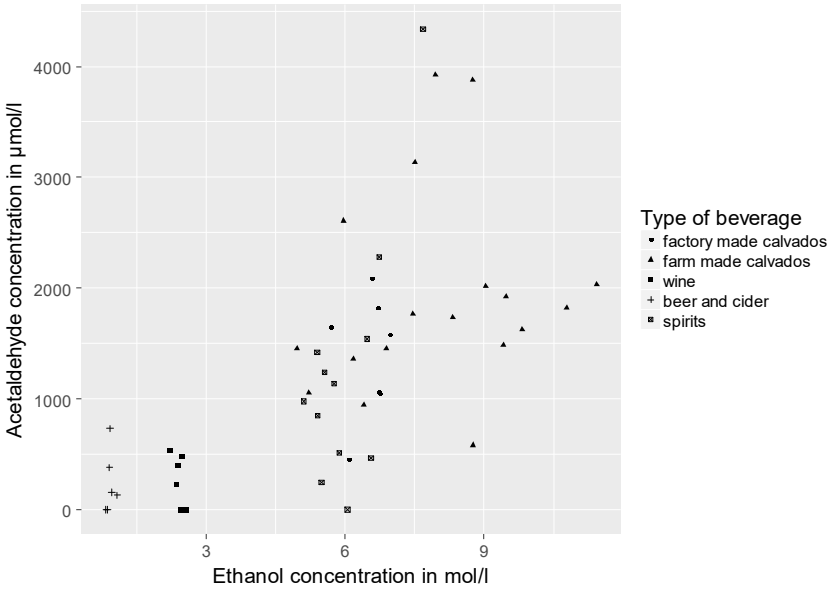

Fig. 2 Scatter plot showing acetaldehyde and ethanol concentrations of all measured beverages

Accordingly, when adjusting acetaldehyde concentration by ethanol concentration we found a significant difference only between calvados and wines. When adjusting for ethanol concentrations the mean acetaldehyde concentrations in farm made calvados and factory-made calvados were 18.81 mg/100 ml pure ethanol and 16.12 mg/100 ml, respectively. (fig. 3).

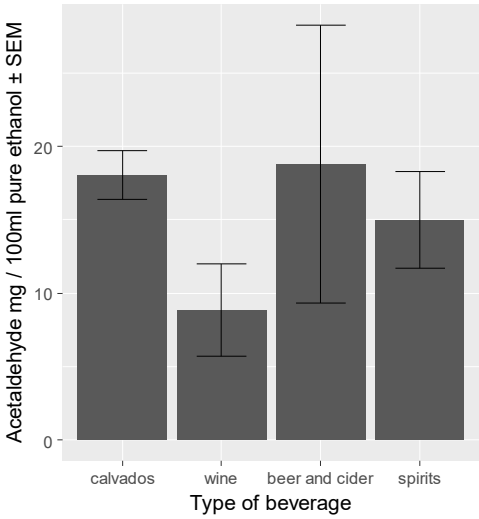

Fig. 3 Acetaldehyde content in beverages adjusted for ethanol concentration (mean ± SEM). There is a significant difference between the calvados and wine groups ($p < 0.05$).

A single sip of a strong alcoholic beverage causes exposure to carcinogenic concentrations of acetaldehyde in the oral cavity

We found significantly higher salivary acetaldehyde concentrations at 30 seconds after sipping calvados vs. 40% ethanol beverage both without and with previous alcohol ingestion (258 ± 89 vs. 122 ± 49 and 215 ± 108 vs. 128 ± 55, calvados vs. ethanol, without and with previous alcohol ingestion, µM ± SD, $p < 0.05$, fig. 4). No difference in acetaldehyde concentration was observed between groups at 2, 5 and 10 minutes. At 2 minutes the mean acetaldehyde concentration ranged from 159 ± 66 to 192 ± 67, µM ± SD. Peak mean acetaldehyde concentrations were measured at 30 seconds after sipping calvados, and at 2 minutes after sipping 40% ethanol, where after the acetaldehyde concentrations decreased with time. When calculating area under the curve for acetaldehyde exposure, no significant differences were observed between groups.

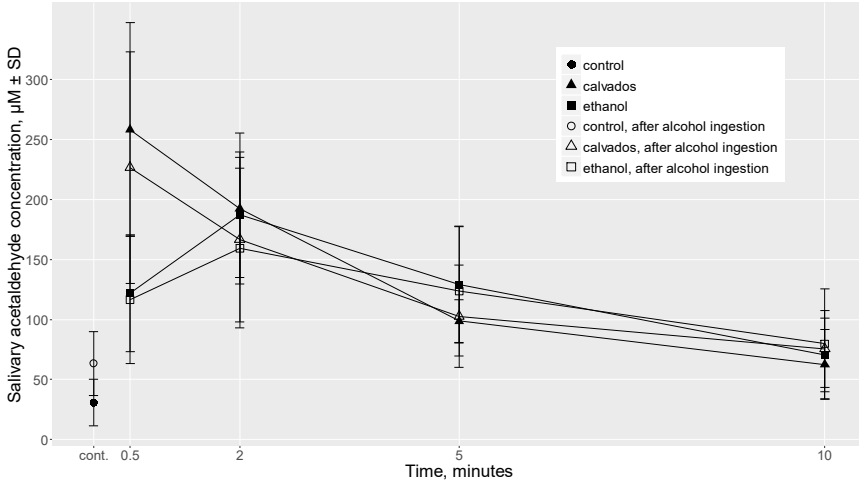

Fig. 4 Acetaldehyde concentration in saliva (mean ± SD). There is a significant difference at 0.5minutes between calvados and ethanol ($p < 0.05$).

Acetaldehyde and ethanol concentrations ± SD were higher in the control samples after alcohol ingestion than without it. (55 ± 32 vs. 27 ± 21, µM ± SD, and 12 ± 7.7 vs 2.7 ± 1.6, mM ± SD, with vs. without previous alcohol ingestion, acetaldehyde and ethanol concentration, $p < 0.05$). Apart from these control samples alcohol ingestion did not result in any significant differences in salivary acetaldehyde concentrations.

Salivary ethanol concentration was over the upper limit of detection of the gas chromatograph in all 30-s and 2-min samples. At 5 minutes mean ethanol

concentration ranged from 83-106mM, with no significant differences between groups. At 10 minutes ethanol concentration was significantly higher after alcohol ingestion (34 ± 14 and 34 ± 13, mM ± SD) than without it (18 ± 6.1 and 17 ± 11, mM ± SD), calvados and ethanol, $p < 0.05$.

Reducing carcinogenic acetaldehyde exposure in the achlorhydric stomach with cysteine

The cysteine capsules delayed the release of L-cysteine for 10-15 minutes, after which L-cysteine was rapidly dissolved. (fig. 5) Powdered L-cysteine was dissolved 100% in 5 minutes.

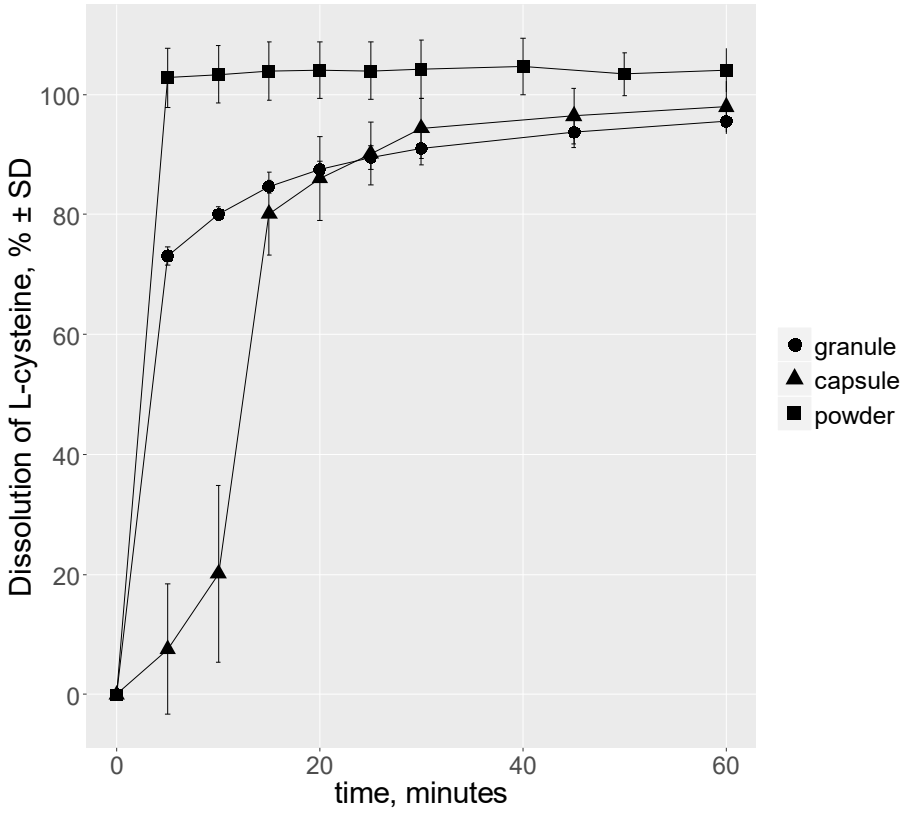

Fig.5 Dissolution of L-cysteine capsules, L-cysteine granules without the capsule shell and L-cysteine powder (dissolution % ± SD)

No changes in gastric juice pH were found between placebo and cysteine administrations, and no significant change in pH was observed during the test. Mean pH ± SD was 6.9 ± 0.7.

The average acetaldehyde concentration of all samples was 2.6 times higher with placebo vs. cysteine (13 vs. 4.7 µM, $p < 0.05$, n=7). The area under the curve (AUC), for the 5 subjects with data up to 40 minutes, was also significantly larger with placebo than with cysteine (531 vs 197 µM × min, $p < 0.05$, n = 5, fig. 6).

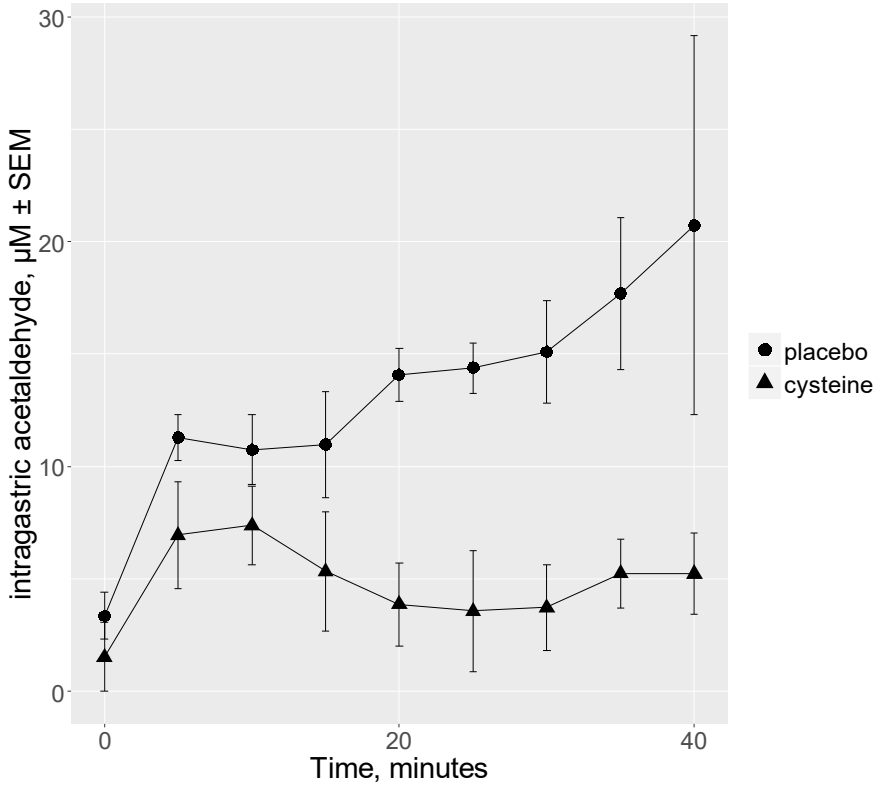

Fig. 6 Acetaldehyde concentration (µM ± SEM) after intragastric ethanol (0.3g/kg body weight) and placebo or cysteine (200mg) administration in 5 subjects with atrophic gastritis.

No significant differences in ethanol concentration existed between cysteine and placebo treatments. Ethanol concentration decreased steadily throughout the experiment (fig. 7). One volunteer reported slight joint pain after placebo, otherwise no side effects were observed.

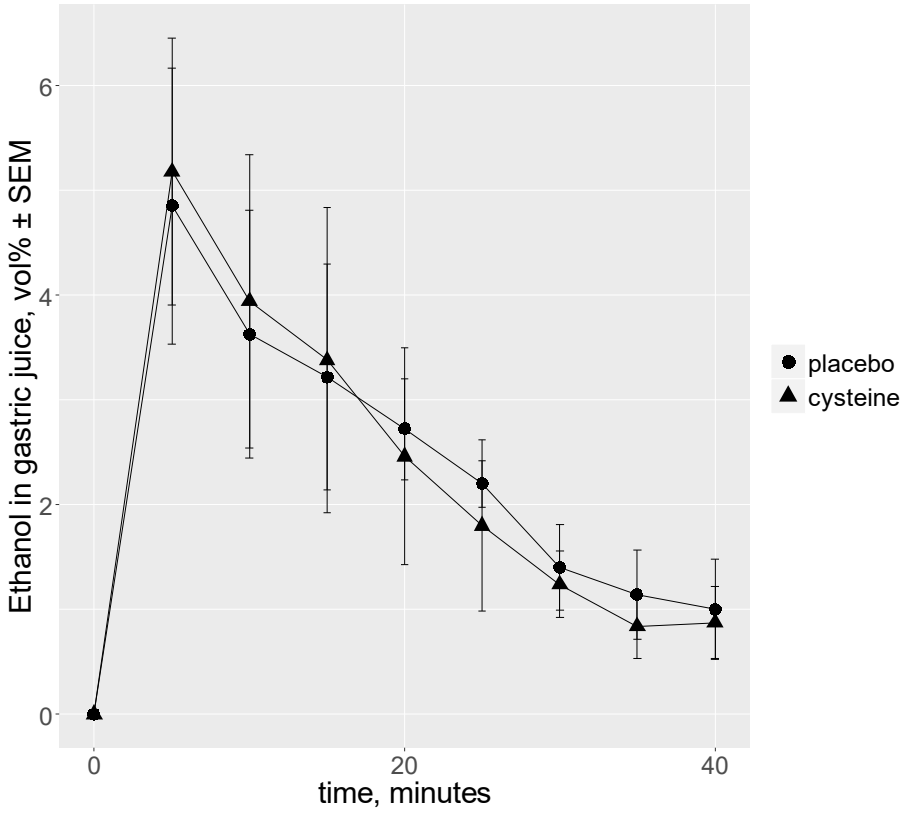

Fig. 7 Ethanol concentration in vol% ± SEM after intragastric ethanol (0.3g/kg body weight, diluted to 15 vol% in water) and placebo or cysteine (200mg) administration in 5 subjects with atrophic gastritis.

L-cysteine was detected in samples following administration of the cysteine capsules (fig. 8.) No cysteine was detected after placebo. We found no significant correlation between cysteine concentration and acetaldehyde concentration ($r = -0.12$, $p = 0.34$)

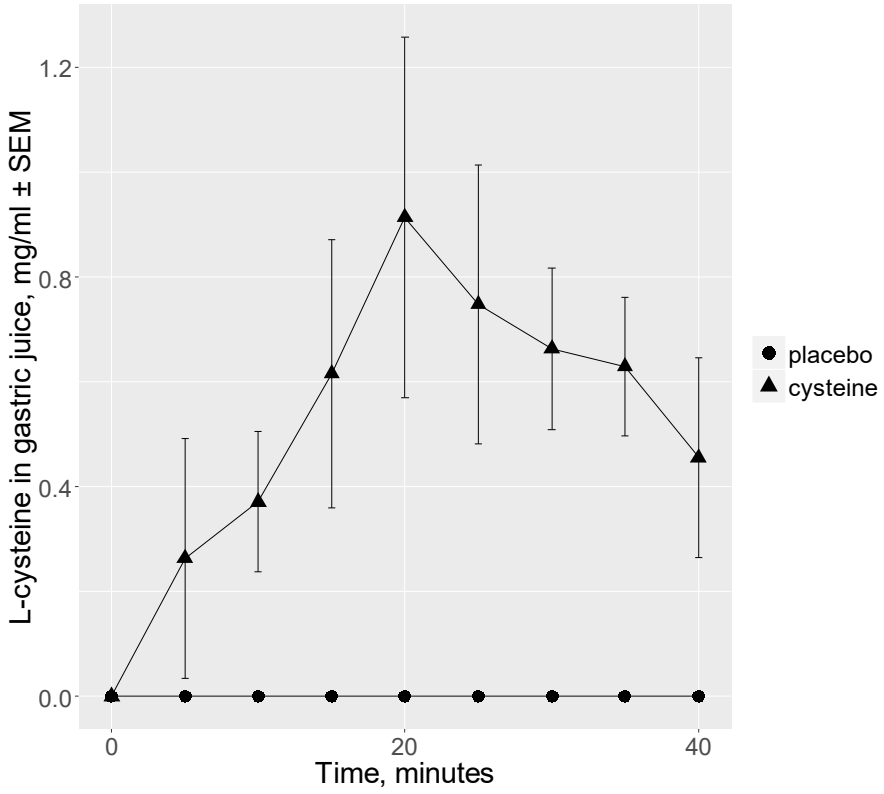

Fig.8 Mean L-cysteine concentration ± SEM in gastric juice after intragastric ethanol (0.3g/kg body weight) and placebo or cysteine (200mg) administration in 5 subjects with atrophic gastritis. (N = 4 at 40 minutes due to insufficient sample volume for cysteine analysis in 1 subject).

DISCUSSION

I – Acetaldehyde in alcoholic beverages, with emphasis on calvados.

We found an abundance of acetaldehyde in calvados, and also in other strong alcoholic beverages. Wines, beers and ciders had lower acetaldehyde concentrations. In this sample of beverages acetaldehyde concentration correlated positively with ethanol concentration, and thus the high acetaldehyde concentration found in calvados is to some extent explained by the high alcohol concentration of farm-made calvados. However, this correlation should not be generalized to alcoholic beverages overall, as the manufacturing process largely affects acetaldehyde concentrations in beverages. For example, when making vodka, several rounds of distillation remove most if not all acetaldehyde from these types of beverages, and acetaldehyde levels in these are generally low (Boffetta et al. 2011). Also, sulphites in wines may reduce the amount of acetaldehyde freely detectable by our method by binding it (Liu, Pilone 2000). When adjusting for alcohol content in beverages, wines contained less acetaldehyde. Consequently, when adjusting for total alcohol consumption direct acetaldehyde exposure excluding acetaldehyde produced from ethanol by microbes and mucosa, is greater from calvados compared to wine. Far greater concentrations of acetaldehyde than were found in our measurements have since been found in other alcoholic beverages. Up to 40mM concentrations were found in grappa (Paiano et al. 2014).

Consumption of hot calvados explained 41% of a regional peak in incidence of oesophageal squamous cell carcinoma in north-western France, when adjusting for other risk factors (Launoy et al. 1997). The mean acetaldehyde concentration in calvados in our study was 1780 µM, range 451-3928 µM. This lies well above salivary acetaldehyde concentrations measured after administration of a moderate dose of alcohol (19 – 143 µM, generally below 100µM) (Homann et al. 1997, Vakevainen, Tillonen, Agarwal et al. 2000, Salaspuro, V., Salaspuro 2004). Salivary concentrations of acetaldehyde measured during cigarette smoking are somewhat higher, 261 ± 45.5 µM ± SEM, n=7. Combining drinking and cigarette smoking resulted in acetaldehyde concentrations up to 350-400µM (Salaspuro, V., Salaspuro 2004). Even while accounting for a small dilution of calvados in saliva when sipped and swallowed, the resulting concentrations lie well above those measured during drinking and smoking.

In the study by Launoy et. al., hot calvados was defined as either calvados with coffee, or more seldom calvados as a grog with hot water. Calvados was

seldom consumed alone or cold in this study population. This may explain the emphasis on hot calvados (Launoy et al. 1997). Any possible mucosal thermal damage caused by hot coffee or grog may contribute to any carcinogenic in a beverage taken simultaneously, and cultural drinking habits may contribute in this way to an individual's risk for alcohol related upper digestive tract cancer.

Our study is limited by the low number of samples from beverages other than calvados. The amount of calvadoses sampled – and particularly different farm-made calvadoses – was sufficient in regards to the scope of this study. The findings have been by confirmed in (Lachenmeier, Sohnius 2008), and similar studies on different alcoholic beverages from around the world also show marked variation in acetaldehyde concentrations (Lachenmeier, Kanteres et al. 2009, Boffetta et al. 2011, Paiano et al. 2014).

We concluded that the acetaldehyde found in calvados could result in an increased acetaldehyde exposure and could explain the increased risk associated with consumption of hot calvados in France. To further examine this theory, we devised the experiment detailed in the second publication in this thesis.

II - Salivary acetaldehyde concentration *in vivo* after ingestion of strong alcoholic beverages.

We found very high concentrations of acetaldehyde in saliva immediately after a small sip of strong alcoholic beverage. The differences between calvados, and diluted ethanol were however surprisingly small, and significant only at 30 seconds after the tasting of the beverages. Neither was there any significant difference in the area under the curve for acetaldehyde concentration × time. The mean concentrations 30s after a sip of calvados were up to 7 times higher than measured previously after alcohol ingestion to a BAC of 0.5 ‰. Small but measurable amounts of acetaldehyde were found in the control saliva samples. In previous studies salivary acetaldehyde without drinking or smoking has been zero (Homann et al. 1997, Vakevainen, Tillonen, Agarwal et al. 2000, Vakevainen, Tillonen, and Salaspuro 2001). This discrepancy is readily explained by acetaldehyde produced from the residual ethanol from the test run, and previous experiment as explained in the study design.

In general, the acetaldehyde concentrations were higher than in previous studies on salivary acetaldehyde concentration after alcohol drinking. The ethanol concentrations in saliva immediately after oral administration of the alcoholic beverage are higher than concentrations distributed through the bloodstream. Thus, the ADH-mediated reaction is tilted by an abundance of substrate (ethanol) towards production of more acetaldehyde. Unfortunately,

ethanol concentrations in the 30-second and 2-minute samples were above the upper limit of measurement for our gas chromatograph. The study by (Maejima et al. 2015) is so far the only study to examine salivary acetaldehyde concentrations not after drinking an alcoholic beverage, but after intragastric infusion. Salivary acetaldehyde concentrations, which stemmed completely from ethanol distributed by the blood stream, were markedly low, less than half compared to previous studies with a similar per oral ethanol dose (Vakevainen, Tillonen, Agarwal et al. 2000, Vakevainen, Tillonen, and Salaspuro 2001, Homann et al. 1997).

Acetaldehyde concentration in saliva at 30 seconds after a 5ml sip of calvados, containing 2400µM acetaldehyde was surprisingly low. We know that there is on average 0.8ml residual saliva in the mouth, and that saliva is secreted at a rate of 0.1ml/min when unstimulated, up to a maximum of 7 ml/min when stimulated (Humphrey, Williamson 2001). If we add 5ml of beverage and swish it around in the mouth for 5 seconds as in this study, the beverage is mixed with saliva and diluted by a factor of 0.86, and the resulting acetaldehyde concentration will be around 2100µM. Only thirty seconds after this we measured on average 242µM acetaldehyde in saliva, a decrease that cannot be explained even by assuming dilution by the maximum flow of saliva. Acetaldehyde is soluble in water and in lipids, and some acetaldehyde is presumed to diffuse into mucosal cells, and the bloodstream. At two minutes the acetaldehyde concentration in saliva was similar regardless of acetaldehyde concentration in the tasted beverage. The acetaldehyde concentration decreased along with the ethanol concentration over time. Altogether this study along with the previous studies suggest that a metabolic and kinetic equilibrium between acetaldehyde and ethanol is achieved, and that salivary acetaldehyde concentration in an individual is largely dependent on the prevalent ethanol concentration.

The study is somewhat limited by the small number of test subjects. This model studying direct exposure to ethanol after a sip of alcoholic beverage results in more variation in salivary ethanol concentration than models with ingested ethanol, distributed via the blood to the mouth and other tissues and bodily fluids in a more predictable manner. This was overlooked when planning the experiments. The increased variance might have caused us to miss some differences in actual acetaldehyde concentration between groups. Also, it would have been interesting to examine sipping a solution of acetaldehyde, but as acetaldehyde is poisonous, we refrained from this. Since calvados is meant and approved for human consumption, it was deemed more acceptable having test subjects sip calvados although it contained acetaldehyde. Due to a misjudgement, in the first 2 samples, salivary ethanol concentrations were above the upper limit of detection of the gas chromatograph. This could have been avoided by reserving some saliva for dilution. Also, the amount of saliva given while sampling was not measured,

saliva was collected in a small bottle by spitting – repeatedly if necessary – until enough was available for pipetting 450µl for analysis. Our results were confirmed in a similar study using several beverages with different acetaldehyde concentrations (Lachenmeier, Monakhova 2011), and also in a study comparing ALDH2 deficient and proficient individuals (Helminen et al. 2013).

We concluded that acetaldehyde in alcoholic beverage is reflected onto salivary acetaldehyde concentration for a very short time immediately after drinking, and acetaldehyde concentration rapidly decreases to a level which is dependent on ethanol concentration. In a normal drinking situation, where repeated sips are taken over time this small peak in acetaldehyde exposure is repeated, and may be of significance. An equally important finding was that after such a sip, high levels of ethanol, and consequently acetaldehyde remain in saliva for at least 10 minutes, and probably up to 25 minutes, as shown by acetaldehyde present in the control samples. We were first to discover that repeated sipping could account for the major part of local acetaldehyde exposure (Nieminen, Salaspuro 2018). In a normal drinking situation, the alcoholic beverage is swallowed repeatedly, and presumably the exposure to high ethanol and acetaldehyde concentrations continue for some undetermined time. This is possibly one of the reasons for the increased risk for cancer caused by alcohol consumption manifesting especially in the upper digestive tract.

III - Eliminating acetaldehyde from stomach using cysteine

We found comparatively low concentrations of acetaldehyde in gastric juice after infusing a small dose of alcohol in test subjects with achlorhydric stomach due to atrophic gastritis. L-cysteine capsules were able to further reduce acetaldehyde concentration in gastric juice. The difference in acetaldehyde concentration was significant from 20 minutes until 40 minutes. According to dissolution tests on the cysteine capsules, the cysteine is released only after 5 - 10 minutes, which explains to some extent why acetaldehyde concentrations were similar in the treatment and placebo groups up to 15 minutes. From 45 minutes onward, we were unable to acquire gastric juice samples from most patients, probably due to stomach emptying and statistical power was diminished. According to gastric juice cysteine measurements the selected dose of 200mg L-cysteine / capsule was adequate, and the L-cysteine was released in a controlled rate. The subjects were fasting during this experiment, and lied on their left sides, to delay gastric emptying. We don't know how well the cysteine would perform in a situation where subjects are standing, maybe even dancing, and drinking alcohol continuously. L-cysteine would probably be driven to the small intestine rapidly.

Our results were confirmed later in two studies using similar L-cysteine releasing capsules (Acetium, Biohit Oyj., Helsinki, Finland). In Japan, 10 ALDH2-deficient and 10 ALDH2-proficient volunteers were studied. Achlorhydria was established with a 7-day regimen of rabeprazole 10mg twice a day. L-cysteine capsules were able to significantly reduce acetaldehyde concentrations in gastric juice after ethanol infusion for up to 2 hours (Maejima et al. 2015). In Sweden 7 subjects with atrophic, achlorhydric gastritis were studied. L-Cysteine capsules were again able to reduce gastric juice acetaldehyde concentrations after intragastric infusion of ethanol. The elimination of acetaldehyde by binding to L-cysteine was further confirmed by detection of elevated MTCA in gastric juice after giving L-cysteine capsules compared to placebo (Hellstrom et al. 2017).

We showed that L-cysteine capsules can be used to reduce exposure to acetaldehyde in stomachs of patients with achlorhydric atrophic gastritis during alcohol exposure. Exogenous ethanol can lead to acetaldehyde production in achlorhydric stomach, but there is also evidence for endogenous ethanol production in achlorhydria due to cimetidine use (Bode et al. 1984), and a slight increase in gastric juice ethanol and acetaldehyde concentrations were detected after intragastric glucose infusion in some patients with atrophic achlorhydric gastritis (Vakevainen et al. 2002). A similar dose of L-cysteine as used in this study would probably be sufficient to bind any endogenously formed acetaldehyde.

It remains to be determined whether L-cysteine capsules can be used to prevent incidence of gastric cancer. The optimal regimen of L-cysteine capsules is unknown and the overall health impact of such treatments is unknown. Although gastric cancer is among the most common causes of cancer death worldwide, the lesions take long time to form, and intervention studies would require exceedingly large study populations and long follow-up times. A population with severe atrophic gastritis, achlorhydria, and ALDH2-deficiency would be an interesting place to start.

Further prospects

Oral microbial and mucosal capacity for production of acetaldehyde from ethanol is high, and elimination of acetaldehyde by oxidation via ALDH is low. This allows for accumulation of acetaldehyde in saliva. The role of ADH in regulating local acetaldehyde exposure is of interest, as the enzymatic reaction is actually favoured towards production of ethanol in hepatic ADH (Deetz et al. 1984). Furthermore, Yin et. al showed in 1984 that the polymorphism in the ADH1B gene, with the ADH1B*2 allele resulting in faster ethanol oxidation

in vitro also increases the rate of acetaldehyde reduction to ethanol *in vitro* in ADH1B isolated from liver. (Yin et al. 1984). However, ADH1B expression was not detected in tongue or gingival mucosa, whereas ADH4 was (Dong et al. 1996). Acetaldehyde reduction in mucosal ADH:s have not been studied, and research in humans has focused on the reaction towards acetaldehyde, as the main function of ADH and ALDH in the alcohol drinking human is to metabolize ethanol to acetaldehyde and further to acetate. Ethanol oxidation by ADH is inhibited by the substrate, acetaldehyde. In liver acetaldehyde is effectively removed by ALDH, and substrate inhibition is of little or no significance. It is possible that the physiological role of ADH in digestive tract mucosa is to reduce acetaldehyde to ethanol, which can then be transported to the liver for elimination. The role of ADH in regulation of acetaldehyde concentration in the upper digestive tract warrants further research in light of this hypothesis, and especially considering the association of ADH1 polymorphisms with upper digestive tract cancer risk, as discussed in the review of the literature.

From our finding that acetaldehyde in calvados is rapidly removed from saliva it appears that mechanisms for acetaldehyde removal in the upper digestive tract are quite effective, only impaired by exposure to the copious amounts of ethanol available in saliva when drinking. The significance of acetaldehyde in foods without ethanol, or with low ethanol concentrations is not known.

High acetaldehyde concentrations can be found in fruits and yoghurts, yet no compelling epidemiological evidence exists for the carcinogenicity of fruits or yoghurts. Acetaldehyde has been found in citrus juices in concentrations up to 5000µM (Lund et al. 1981), and in ripening apples rising from 1800 µM to 15000µM (Karaoulanis, Dilley 1993). In a large German survey clearly lower acetaldehyde concentrations were found, where the maximum acetaldehyde content in an apple was 2.39mg/kg (~50µM) and in a banana 18.27 mg/kg (~400µM) (Uebelacker, Lachenmeier 2011). Acetaldehyde is the compound that contributes most to the typical flavour of yoghurt, and good flavoured yoghurt is said to result when "proper" levels of 500-900µM are achieved (Cheng 2010). In the German survey acetaldehyde concentrations in yoghurts ranged from 2.40mg/kg to 17.42mg/kg (~50 to 400µM) (Uebelacker, Lachenmeier 2011). Consumption of fermented dairy food was associated with a decreased overall cancer risk in a recent meta-analysis, including oesophageal cancer and oropharyngeal cancer (Zhang, K. et al. 2018). Consumption of yoghurt, from less than once a week to daily was associated with a decreased risk for upper aerodigestive tract cancer, with OR:s ranging from 0.67 to 0.73 (Kawakita et al. 2012). There is one study from Iran where daily consumption of yogurt was associated with an increased risk for gastric cancer (OR 16.26; 95%CI 2.10 - 125.73) (Somi et al. 2015). Consumption of fruit is associated with a decreased risk for oral (Pavia et al. 2006),

oesophageal and gastric cancer (Abnet et al. 2015). Overall there is little evidence that acetaldehyde is carcinogenic to humans when not associated with consumption of alcoholic beverages.

Future topics for research suggested are developing and using markers of acetaldehyde exposure, such as the acetaldehyde-DNA adduct N^2-ethylidenedeoxyguanosine to estimate exposure of DNA to acetaldehyde from different sources, whether through metabolism from ethanol, or directly from acetaldehyde in food, beverages, and cigarette smoke. Systematic measurement of ethanol and acetaldehyde content in foods is suggested in order to better asses the acetaldehyde exposure from acetaldehyde in non-alcoholic foods and beverages. These markers could also be used to further study the use of L-cysteine in acetaldehyde elimination.

SUMMARY

The main findings in this thesis are:

1. Acetaldehyde is found in different alcoholic beverages, and in some, at high concentrations which may contribute to mucosal acetaldehyde exposure.
2. Acetaldehyde present in alcoholic beverages contributes to acetaldehyde exposure in the oral cavity after a single sip of beverage. After each sip, ethanol remains in saliva for up to ten minutes in concentrations that allow local formation of mutagenic acetaldehyde. Thus, the major part of acetaldehyde exposure associating with alcohol drinking stems from rapid oxidation of ethanol in the mouth.
3. Slow release L-cysteine capsules can be used to reduce acetaldehyde concentration in achlorhydric gastric juice after intragastric ethanol infusion.

There is compelling epidemiological, genetic and biochemical evidence that local acetaldehyde exposure from ethanol oxidation in the upper digestive tract contributes to the development of cancer. This thesis provides further mechanistic insight in the kinetics of acetaldehyde formation and elimination in the mouth and introduces the concept of eliminating acetaldehyde in achlorhydric stomach by binding it to L-cysteine.

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